

Evolutionary Biology

Linking genotypic and phenotypic changes in the *E. coli* Long-Term Evolution Experiment using metabolomics

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Abstract

1 Abstract

Changes in an organism's environment, genome, or gene expression patterns can lead to changes in its metabolism. The metabolic phenotype can be under selection and contributes to adaptation. However, the networked and convoluted nature of an organism's metabolism makes relating mutations, metabolic changes, and effects on fitness challenging. To overcome this challenge, we use the Long-Term Evolution Experiment (LTEE) with *E. coli* as a model to understand how mutations can eventually affect metabolism and perhaps fitness. We used mass-spectrometry to broadly survey the metabolomes of the ancestral strains and all 12 evolved lines. We combined this metabolic data with mutation and expression data to suggest how mutations that alter specific reaction pathways, such as the biosynthesis of nicotinamide adenine dinucleotide, might increase fitness in the system. Our work provides a better understanding of how mutations might affect fitness through the metabolic changes in the LTEE and thus provides a major step in developing a complete genotype-phenotype map for this experimental system.

eLife assessment

This study presents **convincing** evidence that metabolite levels in *Escherichia coli* bacteria from a long-term evolution experiment have changed in consistent ways, which in turn can be explained by recurrent mutations in regulatory genes that affect enzyme expression levels. The use of high-resolution mass spectrometry measuring bulk metabolite levels, in combination with existing gene expression and DNA sequencing datasets provides **valuable** information linking changes in an organism's genome, transcriptome, and metabolome.

2 Introduction

Adaptation is the process by which organisms become fitter for the environment in which they live. While rooted in genetic changes, adaptive evolution can be studied at many levels of molecular and organismal phenotypes [1]. An organism's metabolism is one of the fundamental molecular phenotypes supporting its life. It is no surprise, then, that adaptive evolution can proceed through changes in metabolism. Studies have found evidence of adaptation by comparing the metabolomes of closely related organisms. An analysis of metabolic differences between species of *Drosophila* found that these differences can affect lifespan and are sex-specific [2]. In prokaryotes, studies suggest that the genes involved in secondary metabolite production evolve quickly and produce large varieties of phenotypes. For example, strains of *Pseudomonas aeruginosa* that can produce surfactants can better mitigate oxidative stress compared to those that cannot [3]. *E. coli* have been shown to evolve repeatable metabolic changes when grown under selection to increase their biomass production and metabolism is often targeted for directed evolution when engineering organisms for industrial uses [4]. *E. coli* have also been shown produce coexisting subclones that occupy particular metabolic niches in a lab setting [5]. Despite these natural and laboratory-based examples, relating genetic, metabolic, and fitness changes is challenging because complete sets of molecular data are often unavailable.

Laboratory evolution experiments provide ample opportunities to study metabolic evolution. The Long-Term Evolution Experiment (LTEE) is an experimental evolution system where 12 replicate populations of *E. coli* (designated as Ara+1:6 and Ara-1:6, hereafter referred to as A-1:6 and A+1:6) are propagated in a carbon-limited medium with a 24-hour serial transfer regime [6]. Begun in 1988, the LTEE populations have evolved for more than 75,000 generations, making it the longest-running experiment of its kind. Over time, all 12 populations have continued to adapt to this environment. For example, relative fitness (measured as growth rate relative to the ancestral strain) has increased in parallel across the 12 populations [7]. Cellular size in each population has also increased [8, 9]. Parallel genomic changes in the LTEE are well characterized, and while some genes are commonly mutated across the replicate populations, most mutations are unique to each line [10–13]. Despite the variability at the genetic level, the evolved populations display similar gene expression profiles [14, 15]. In principle, mutations and changes to gene expression could affect the proteome by altering the function of a protein, the amount of protein produced, or the conditions under which it is translated. Because proteins are the biological catalysts for cellular chemistry, mutations and gene expression changes may affect the metabolome. In the LTEE, several examples of metabolic changes have been documented. The A-3 population has uniquely evolved the ability to metabolize citrate under aerobic conditions [16, 17] and A-2 has produced unique, coexisting ecotypes [18–20]. These ecotypes exhibit growth-phase dependent frequencies and differ in their ability to metabolize acetate, with one ecotype (the L ecotype) specializing on glucose and the other (the S ecotype) on acetate. The transposable-element induced loss of the *rbs* operon occurred early and prevents the evolved lines from growing solely on ribose [21]. Reduced ability to grow on maltose has occurred through other mechanisms [22–24]. Despite glucose being the only source of carbon provided, significant changes in the ability to grow on other carbon sources have occurred [25]. In particular, the mutator lines exhibit reduced performance on carbon sources other than glucose. The evolved lines have increased rates of glucose uptake as well as altered flux through some central carbon metabolism pathways [26].

While specific examples of how genetic changes result in metabolic changes in the LTEE (citrate, acetate, maltose, ribose) exist, a more general survey of the metabolome is lacking. Such a survey would allow a better understanding of the effects of genetic and expression changes on metabolism and provide the ability to test hypotheses from previous data. By

integrating genomic [10], expression [15], and metabolic datasets, we can relate mutations to expression changes and their effects at the metabolic level. We provide ample evidence for hypotheses that specific genetic changes may exert their fitness-altering effects by changing gene expression and, ultimately, metabolism.

3 Results

3.1 Survey of metabolic changes in the LTEE

We used liquid chromatography coupled mass-spectrometry (LC/MS) to scan a broad mass range using both positive and negative ionization modes to survey the metabolomes of both ancestral strains and clones from each of the 12 evolved lines of the LTEE at 50,000 generations. We sampled two time points, 2 and 24 hours, to represent exponential and stationary phase samples, each with two biological replicates. It is important to note that the 2 hour time point captures the evolved lines, including A-3, during their growth on glucose. Additionally, the evolved lines reach stationary phase after about 4 hours of growth, with A-3 experiencing growth that levels off slowly over around 14 hours [27]. At our 24 hour time point, the evolved lines (except A-3) have been in stationary phase for around 20 hours. The differences between the early, mid, and late stationary phases are unknown. Metabolite abundances were estimated using normalized peak areas relative to standards as described in the Data processing and description. Many metabolites are detected in both ionization modes, making it unclear which mode most accurately represents the abundance of the compound in a sample. As a result, we used the combination of a compound and the ionization mode it was detected in as a feature of the data.

We begin by surveying the data in a metabolome-wide manner. Because *E. coli* exhibits large physiological differences across growth phases [28–30], we expected comparisons within a growth phase to be more similar than comparisons across growth-phases. Indeed, the distribution of within growth phase correlations of metabolite abundances is higher than the distribution of across growth-phase correlations (Fig. S1A). Because growth-specific differences have the potential to drown out signals of adaptation, we performed all subsequent analyses in a growth phase-specific manner.

To identify large scale differences in metabolite abundances, we performed a principal component analysis (PCA) of metabolite abundances in a phase-specific manner. In the exponential phase, the PCA strongly separates A-3 from the other samples (Fig. 1A). This is possibly due to the unique ability of A-3 to metabolize citrate. Citrate is present in the medium because it was originally added as a chelating agent. However, mutations in A-3 have allowed it to metabolize citrate under aerobic conditions, giving it access to additional carbon that the other lines do not have [16, 31]. Half of the evolved lines have a mutator phenotype and have at least an order of magnitude higher number of fixed point mutations than the non-mutators [10]. Despite the large differences in the number of mutations that have accumulated, we do not observe a strong effect of mutator status on the separation between evolved lines in the PCA. This is consistent with previous observations of a minimal effect of mutator status on changes in gene-expression patterns [15]. The first two principal components are dominated by contributions of nucleoside monophosphates, amino acids, and compounds involved in carbon metabolism, such as glucose and succinate (Fig. S3A-B).

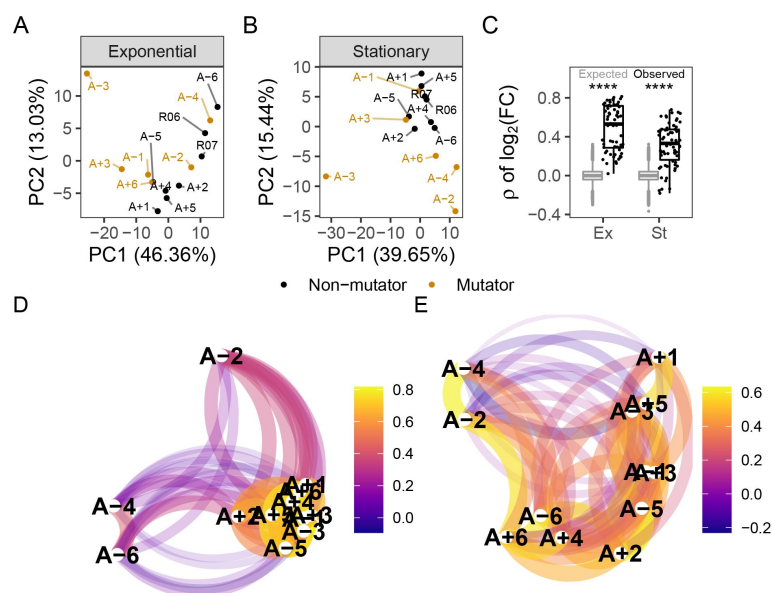


Figure 1.

(A-B) Principal component analysis based on $\log_{10}(\text{mean normalized peak area})$ separated by growth phase. R06 and R07 are the ancestors (REL606 and REL607). For this figure, the combination of ionization mode and metabolite was treated as a feature of the data. **(C)** Pairwise Spearman's correlations based on $\log_2(\text{fold - change})$ relative to the ancestor. The black boxes and points indicate the observed correlations, the grey boxes indicate correlations calculated after 100,000 randomizations of fold-changes within each line. p-values indicate the results of a two-tailed t-test between the observed and expected distributions. **** indicates a p-value ≤ 0.0001 .

(D-E) The observed correlations from C plotted in a network manner. D is the exponential phase

and E is the stationary phase. Lines are clustered based on similarity and the color of the line connecting two points indicates the strength of the correlation.

Similar to the exponential phase, A-3 separates from the rest of the evolved lines in a PCA of metabolites in the stationary phase (Fig. 1B). The primary driver of variation in the first principal component (PC1) is the relative abundances of compounds in A-3 (Fig. S4A). In addition to A-3, A-2 also shows a higher degree of separation from the other lines. Though this experiment was performed with single clones, the 12 flasks in the LTEE are not isoclonal but instead consist of competing subclones that sometimes coexist for extended periods [12]. A-2 is the best-understood example, consisting of two major subclones, L and S [18–20]. The observed separation might be because the A-2 clone in this experiment comes from the L ecotype, which does not grow as well on acetate compared to the other lines. In contrast to exponential phase PCA, mutator status appears to be a driving factor of PC2 in the stationary phase (Fig. S4A-B) with mutator lines having lower abundances of nucleoside monophosphates relative to non-mutators during stationary phase. What this is indicative of is unclear.

A common theme in studies of LTEE is the high degree of parallelism observed at the genetic [10], gene expression [15], and fitness levels [32]. For example, genes involved in flagellar biosynthesis and amino acid metabolism are commonly mutated across the evolved lines [10] with associated changes in expression levels [15]. To examine the extent of parallel changes in metabolites, we calculated the ratio of the peak area in an evolved line to the peak area in the ancestor for each compound after averaging biological replicates. Pairwise Spearman's correlations using these ratios are significantly higher than expected when comparing the observed distribution to a distribution generated by randomizing fold-changes within a sample prior to calculating correlations (Fig. 1C, $p < 0.001$, t-test). In the exponential phase, the lowest correlations were mainly between a pair involving one of A-2, A-4, or A-6, and another evolved line (Fig. 1D). The correlations of metabolite abundances across evolved lines are more similar in the exponential phase (Fig. 1D) than in the stationary phase (Fig. 1E) (t-test comparing the observed exponential and stationary phase correlations in Fig. 1C, $p < 0.001$). It may be that the strategies of metabolite usage and abundance in the rapidly growing exponential phase are more similar across lines than the strategies of survival are in the stationary phase or that there is less selection on the

metabolome during the stationary phase. Further studies would be required to understand this phenomenon.

To further quantify the extent of shared changes, we considered the difference between the number of shared changes that were observed and expected to be observed by chance. We approximated an expected distribution of shared changes using a Sum of Independent Non-Identical Binomial Random Variables (SINIB) method [33], essentially asking what the chance of repeatedly observing the same change in an increasing number of evolved lines is. For more details on this method, see section 5.1.5. For metabolic features (the combination of a compound and the ionization mode it was detected in) that shared alterations in only a few lines (generally six or less), we cannot suggest selection on these changes because we see fewer or as many changes as expected (Fig. S5, Fig. S6). These may represent metabolites that negatively affect fitness when altered. On the other hand, we observe more changes shared in a higher number of evolved lines than expected. These shared changes may be beneficial, but this would need to be clarified by additional experiments. By combining genomic, expression, and metabolic datasets, we can better explore the nature of these changes and how they may impact fitness.

3.2 Increased NAD may facilitate higher energy demands during adaptation

In the LTEE, mutations to the *nadR* gene have occurred in all of the evolved lines and appear to have been under strong selection to improve fitness [10, 34]. NadR is a dual-function protein that negatively regulates genes involved in the synthesis of nicotinamide adenine dinucleotide (NAD) and has its own kinase activity [35, 36].

NAD and its phosphorylated derivative NADP function as electron carriers and are necessary for many catabolic and anabolic pathways in the cell. While untested, hypotheses for how *nadR* mutations might increase fitness involve derepression of its target genes and increases to intracellular NAD; this may aid in shortening the lag phase and modulating redox chemistry [34, 38, 39]. The abundance of NAD and related compounds in a cell can modulate reaction rates and other processes [36, 40]. We had previously shown that mutations in *nadR* in the evolved lines lead to consistent upregulation of its target genes [15] (Fig. 2). Here we test if there are higher abundances of NAD and NAD-related metabolites in the evolved lines consistent with changes observed at the genetic and gene expression levels.

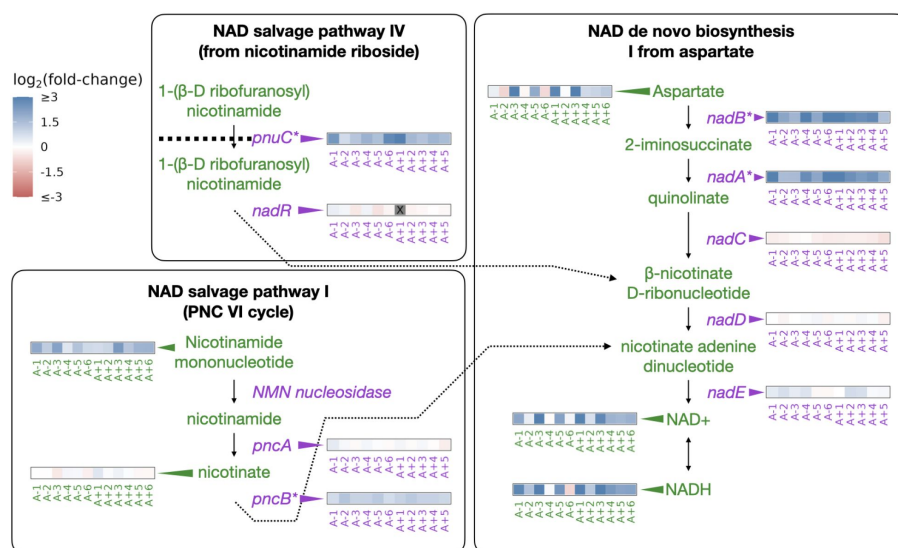


Figure 2.

Depiction of three pathways (bold-faced text) that contribute to NAD abundances in the cell. Graphics and pathway names are adapted from the EcoCyc database [37]. All data represents exponential phase measurements. Genes that code for enzymes are shown in purple and metabolites in green. Heatmaps positioned to the right of gene names show the fold-change in expression relative to the ancestor (data from [15]). Grey spaces (also marked with an X) in gene expression heatmaps

represent evolved lines where that gene contains an indel or is deleted. Asterisks indicate genes that are transcriptionally regulated by NadR. Heatmaps positioned to the left of metabolite names show changes in metabolite abundance relative to the ancestor. PnuC transports compounds into the cell. Each heatmap represents one ionization mode, but a mixture of positive and negative ionization mode data is shown depending on which mode a compound was detected. See [Fig. S7](#) for complete data.

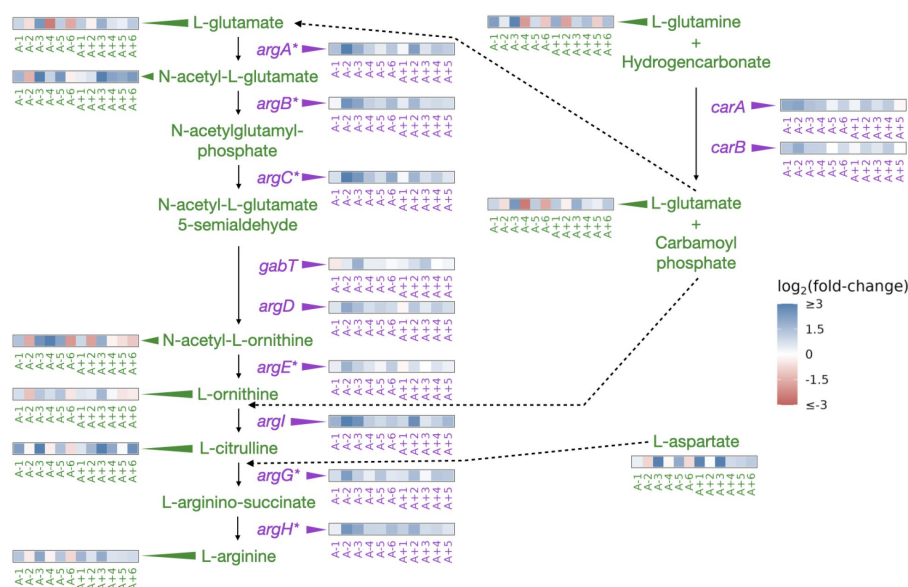
The genes regulated by NadR participate in specific reactions along NAD synthesis pathways. We detected some of the compounds in these pathways as well as both oxidized and reduced forms of NAD and NADP ([Fig. 2](#)). Compared to the ancestors, both redox states of NAD and NADP are almost universally increased in the evolved lines during the exponential phase ([Fig. 2](#), [Fig. S7](#)), with median fold-changes of 3.32 and 4.65 for NAD and NADH, respectively. NADP is generated through phosphorylation of NAD by NadK [36, 41] and saw median fold-changes of 2.34 and 3.54 for NADP and NADPH, respectively. Because NADP is generated from NAD, and each exists in two oxidation states, we expected all four compounds to see similar increases within an evolved line. Indeed, increases in the various redox and phosphorylation states were consistent within an evolved line ($0.77 < R < 0.97$, [Fig. S7B](#)). Aspartate, the starting point for NAD synthesis, was also increased in most of the evolved lines, with a median fold-change of 1.94. Nicotinamide mononucleotide, which can be used to make NAD, was consistently increased, with a median fold-change of 2.51. In the stationary phase, most of these patterns remain unchanged except for aspartate, which is generally lower in abundance compared to the ancestor ([Fig. S7A](#), [S3C](#)). These results suggest that the end product of the mutations in *nadR* is increased intracellular NAD.

3.3 Derepression of Arginine biosynthesis at genetic, gene expression, and metabolic levels

Similar to *nadR*, another consistent target of mutations in the LTEE is a transcriptional repressor of genes involved in arginine biosynthesis *argR* [10]. ArgR, along with arginine, negatively autoregulates the arginine biosynthesis pathway by downregulating the participating genes when arginine is abundant [42]. As an amino acid, the primary role of arginine is in protein synthesis. However, various reactions produce and consume arginine; both its synthesis and the regulation of the enzymes involved are complex (see [43] and the EcoCyc [37] pathway “superpathway of arginine and polyamine biosynthesis”). For example, arginine can be a source of nitrogen and carbon or be used for ATP synthesis [44]. Because of these complications, if mutations in *argR* and the subsequent changes in gene expression do affect this pathway, it is unclear at which step one might observe a change or if that change might be in a different pathway.

Mutations in *argR* are localized to specific regions, potentially disrupting the DNA binding domain, the arginine sensing domain, or interaction domain, which is required for hexamerization of the protein [42]. These mutations in *argR* could reduce its ability to repress its target genes, resulting in their increased expression. Consistent with these expectations, we had previously observed parallel increases in the expression of genes repressed by ArgR ([Fig. 3](#)) [15].

Figure 3.



one ionization mode, but a mixture of positive and negative ionization mode data is shown depending on which mode a compound was detected. See [Fig. S8](#) for complete data and [Fig. S9](#) for line specific data.

We were able to detect 11 compounds involved in the superpathway of arginine and polyamine biosynthesis (Fig. 3, Fig. S8A). Compared to the ancestor, arginine was increased in nine (negative mode) or ten (positive mode) evolved lines and experienced a median fold-change of 1.9 across both ionization modes in the exponential phase. This pattern also persisted in the stationary phase (Fig. S8A). While arginine is increased in both growth phases in most of the evolved lines, other compounds in this pathway show highly variable changes (Fig. S8A). Despite the consistent relationship between mutations, expression changes, and changes in the abundance of arginine, how this might affect fitness is not obvious. Higher intracellular abundances of amino acids could facilitate higher translation rates and promote faster growth. While we do observe that some amino acids show increases in their abundances in most evolved lines in the exponential phase (Fig. S8), further experiments would be needed to confirm if these amino acids are used in protein synthesis or in other pathways.

3.4 Functional changes in the central carbon metabolism

Low carbon availability is the key feature of the minimal medium used in the LTEE, and selection for better use of carbon is a driving force of adaptation in the system. Hence, how mutations and expression changes might affect fitness by altering central carbon metabolism pathways is of interest. The evolved lines have seen significant positive and negative changes in their ability to grow on different carbon substrates. Rather than metabolic specialization, this was found to be due to the accumulation of deleterious mutations that affect the ability to grow on other substrates [25]. Unique changes in A-3 allow it to metabolize citrate under aerobic conditions, granting it access to extra carbon from an unused ecological niche [16, 17]. The evolved lines retain more carbon in their biomass compared to the ancestors [31], and increased glucose uptake rates have been demonstrated [26]. Despite the abundance of molecular data associated with the LTEE, the highly networked nature of the genes and metabolites involved in central carbon metabolism makes relating the various molecular data to each other challenging. In

particular, mass-spectrometry data alone will not allow us to differentiate between sources for compounds that are generated by many reactions. We might overcome this limitation by combining genomic, expression, and metabolic datasets.

We again sought instances of parallel sets of mutations and expression changes that may exert their effects at the metabolic level. Three genes that are key components in the glyoxylate cycle, *aceB*, *aceA*, and *aceK* were commonly upregulated in the evolved lines [15]. Their transcriptional regulators *iclR* and *arcB* are heavily mutated [10], and this is known to be beneficial in the LTEE [17]. The glyoxylate cycle allows the use of acetate as a carbon source by converting it to succinate, which can later be fed into the citric acid cycle [37, 45]. Because these genes had increased expression, the compounds they produce (succinate, glyoxylate, and malate) may be elevated in the evolved lines relative to the ancestor. Succinate and malate were reliably identified in our data, but glyoxylate was not. Unfortunately, acetate was part of the mobile phase of our liquid chromatography setup, thus preventing its accurate quantification. Likewise, most of the molecules in the glyoxylate cycle, including succinate and malate, are also components of the citric acid cycle and can be produced or consumed by other enzymes. Because we cannot distinguish where changes in these metabolites come from, we chose to look at compounds from central carbon metabolism in general, considering compounds from the glyoxylate cycle, glycolysis, and the citric acid cycle.

Overall, 18 compounds from these pathways are detected in our LC/MS assay. Regarding the glyoxylate shunt, malate, aconitate, and succinate were generally elevated in the evolved lines (median fold-change of 5.38, 1.66, and 2.30 respectively (Fig. 4)). Interestingly, the evolved lines appear to have similar or lower amounts of glucose despite having been shown to have increased glucose uptake rates [26]. This discrepancy is likely due to the fact that in [26], glucose uptake rates were calculated by measuring the depletion of glucose in the medium, whereas we measured glucose from inside the cells. Once inside the cell, we suspect that the glucose is quickly used. This is supported by the fact that other downstream glycolysis compounds, like phosphoenolpyruvate (PEP), are generally elevated (median fold-change of 6.72, Fig. 4).

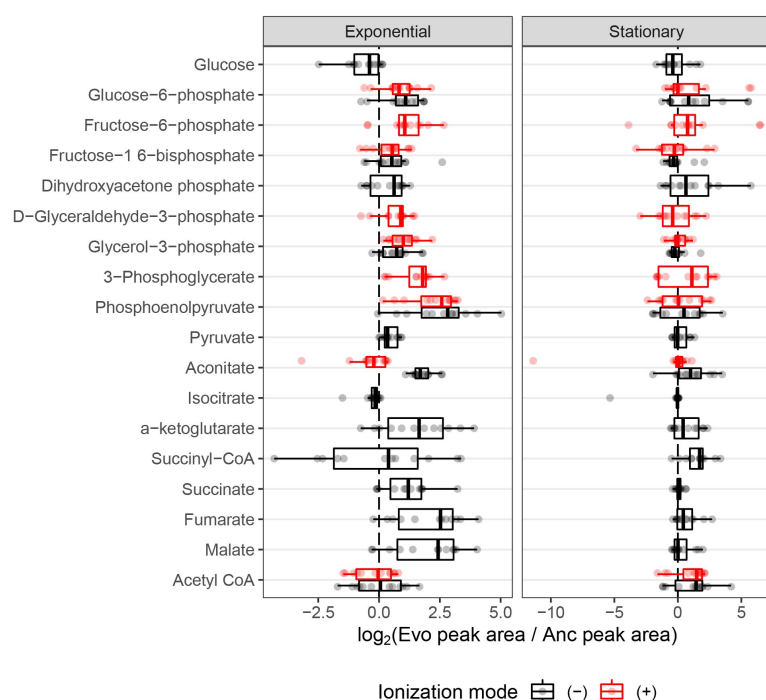


Figure 4.

The distribution of fold-changes relative to the ancestor for compounds involved in carbon metabolism. Red and black indicate detection in positive or negative ionization mode, respectively. Not all compounds were detected in both ionization modes. Compounds are ordered from top to bottom roughly as they occur in glycolysis or other reactions.

An increase in PEP is also consistent with a hypothesis related to increased glucose uptake. It was previously noted that all of the evolved lines contain what are likely inactivating mutations in the gene *pykF*. *pykF* encodes pyruvate kinase I, one of two isozymes that generate ATP by converting PEP to pyruvate [46]. It was thought that inactivating mutations in *pykF* reduce the conversion of PEP to pyruvate, forcing a buildup of PEP, and that because PEP is the energy source for glucose import in the sugar-transporting phosphotransferase system, a buildup of PEP might increase glucose uptake rates. Our data, which shows increases in PEP, lends support to these hypotheses.

The citric acid cycle is a major metabolic pathway that extracts energy via the reduction of electron carriers like NAD. These electrons are shuttled to the electron transport chain by NAD, where they are used to generate ATP. NAD can be reduced at two points during the citric acid cycle, the conversion of alpha-ketoglutarate to succinate and the conversion of malate to oxaloacetate. If the citric acid cycle is running faster in the evolved lines due to their higher growth rates and hence higher energy demands, then more NAD may be required to efficiently shuttle electrons from the citric acid cycle to the electron transport chain. Interestingly, we measured a median fold-change of 3.13 for alpha-ketoglutarate and 5.38 for malate (Fig. 4). Additionally, increases in these compounds are correlated with an increase in NAD compounds within an evolved line (Fig. S10). It may be that increases in NAD allow faster operation of the citric acid cycle. However, alternative hypotheses must be considered.

Overflow metabolism appears to be common in the evolved lines. Overflow metabolism refers to the seemingly wasteful production of metabolites that do not participate in the most efficient form of ATP generation, even when glycolytic substrates and oxygen are plentiful. The Warburg effect [47] and acetate production are examples of this phenomenon in human cancers and *E. coli* [48] respectively. Our observation of the buildup of PEP is an example of overflow metabolism and may lend support to the hypothesis that the evolved lines are using overflow metabolism to gain access to the glycolytic intermediates and using them for other purposes, like as an energy source for sugar import. Experiments using radioactive tracers to study flux through these pathways are needed to confirm any hypotheses.

4 Discussion

The metabolome of a cell is the integration point of an organism's environment, genetics, and gene expression patterns. Metabolism has been shown to contribute to generating adaptive phenotypes [2, 49–51]. However, how mutations affect metabolism through changes in gene expression and ultimately affect fitness is less clear. Specific examples of this have been studied in the LTEE, such as the acquisition of citrate metabolism in A-3 [16, 17]. We used metabolic mass-spectrometry to study other aspects of the LTEE, relating mutations and expression changes to metabolic changes.

This allowed us to lend support to previous hypotheses of how certain mutations affect fitness in the system. For instance, we show how mutations in *nadR* alter the expression of its target genes and increase the intracellular abundance of NAD. It also enables new areas of investigation, such as what role mutations to *argR* and subsequent expression and metabolic changes might mean for the system.

A key limitation of the data presented here is that it contains measurements of 196 metabolites out of the 3755 metabolites currently annotated in the *E. coli* metabolome database [52]. Nonetheless, analysis of this subset of data can reveal some global patterns of

metabolite changes. For example, distinct clustering of A-3 and A-2 in the PCA's suggests that even with a limited sampling of the metabolome, the unique characteristics of these lineages are observable at the metabolic level. Interestingly, while the mutator lines do not form a well-defined group in the exponential phase, their reduced abundances of nucleoside monophosphates in the stationary phase group them together (Fig. 1B). Due to deletions of key biosynthetic genes, the mutators are less flexible than the non-mutators in their ability to grow on different carbon sources [25]. A-6, in particular, has one of the higher mutational burdens and is the least flexible in its ability to grow on different carbon substrates. Pathways with missing or broken genes may cause the shunting of those compounds to different pathways while also starving downstream reactions of the now missing reactants.

Though most studies of the LTEE focus on the activity of the evolved lines in the exponential phase, they spend most of their time in the stationary phase. Experiments studying the evolution of *E. coli* during long-term stationary phase (around three years of culture with no addition of resources) showed that mutations continue to accumulate during this time and that patterns suggestive of adaptive evolution had occurred [53, 54]. While our study chose the latest possible time point for examining the stationary phase (immediately before the transfer to a new flask), differences between early, mid, and late stationary phases may exist. Time course experiments with a finer resolution would reveal details about the specific nature of the stationary phase in the LTEE.

Acknowledgements

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Competing interests

Premal Shah is a member of the Scientific Advisory Board of Trestle Biosciences and is a Director at Ananke Therapeutics. Srujana S Yadavalli consults and collaborates with Designs for Vision Inc. Kyle S. Skalenko is a scientist at Specialty Assays Inc.

5 Supplementary text

5.1 Methods

5.1.1 Cell culture

The A-3 clone used in this experiment is capable of metabolizing citrate, and the clone of A-2 is the L variant [15]. Frozen bacterial stocks were revived by growing them in 5mL LB broth for 24 hours. Following this, 1% of each culture was transferred to 5mL standard LTEE medium for another 24 hours. After 24 hours, these cultures were used to initiate the final experimental cultures. Each clone was grown in 250mL of medium in a 1L flask following standard LTEE protocols. The medium used was standard LTEE medium with the standard

amount of glucose, 25 mg/mL. After 2 hours of growth, 160mL of the sample was removed for exponential phase mass-spectrometry samples. After 24 hours of growth, 40mL of the sample was removed for stationary phase samples. Each line had two independent biological replicates.

5.1.2 Mass-spectrometry sample collection

Cells were collected via vacuum filtration using Millipore Omnipore 0.2 μ m filters (JGWP04700). Sterile plastic petri dishes were placed onto a metal tray on dry ice, and 1.2mL of extraction solvent (40:40:20 acetonitrile : methanol : water +0.5% formic acid) was added to the petri dish to chill. When filtration was complete, the filter was placed cell-side down into the petri dish. The metal tray was moved to wet ice to extract for 20 minutes. After 20 minutes, the acid was neutralized by the addition of 1.07% (final) ammonium bicarbonate, then cell debris was spun down at 14,000 rpm at 4C for 10 minutes, and the clarified extract was transferred to a chilled 2mL tube and stored at -20C. The filter was extracted again by the addition of 0.4mL extraction solvent and sat for 15 minutes. The extract was neutralized, clarified, and consolidated with the first extract and incubated overnight at -20C.

The next day, precipitated protein was spun down at 14,000 rpm at 4C for 10 minutes, and solvents were removed by speed vac for 2 hours at room temperature. Lastly, the sample was concentrated by removing the remaining water by lyophilization. Dried samples were reconstituted in 40 μ L of extraction solvent and submitted for mass spectrometry.

5.1.3 Mass-spectrometry

Mass spectrometry was performed as described previously in [55] at the Rutgers University Cancer Institute of New Jersey metabolomics core facility using a Thermo Q Exactive PLUS coupled with a Thermo Vanquish UHPLC system.

5.1.4 Data processing and description

The raw mass spectrometry data are deposited at the metabolomics workbench under the study ID ST002431 and the code for the analysis at https://github.com/shahlab/ltee_massspec. Normalization of the mass-spectrometry data was performed in two steps. First, raw peak areas were normalized against internal standards as in [55]. After these values were generated, all other data processing steps were performed using the R programming language [56] and the tidyverse set of packages [57]. Code for this section can be found in the document titled data processing.Rmd. Peak areas resulting from the first step of normalization were then taken as a proportion of the total peak area for a single sample to normalize against differences in input amounts.

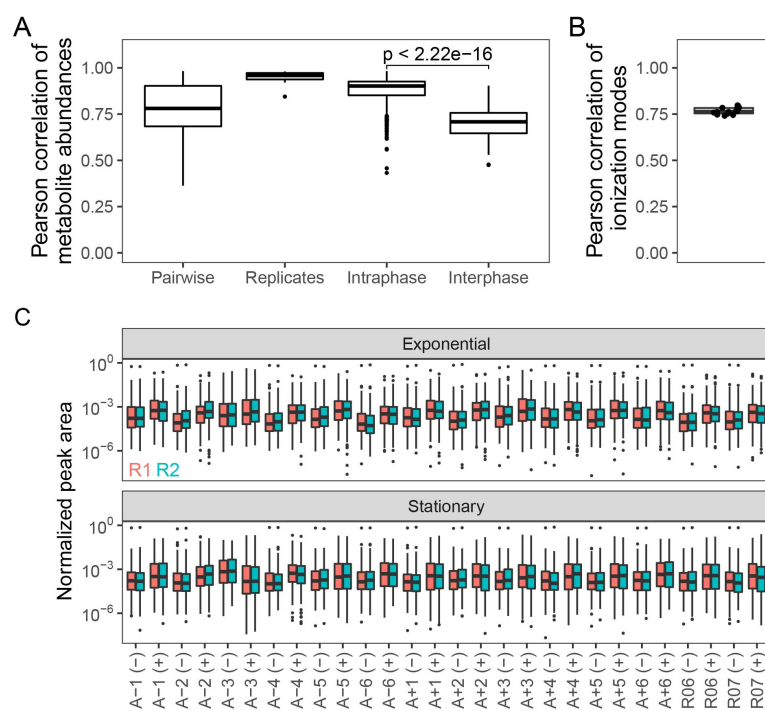
As noted in the text, not all of the 196 compounds were detected in all samples. This varied depending on the compound, line, growth phase, and ionization mode. Overall, 168 compounds were present in all samples in at least one ionization mode, with the remaining 28 compounds being undetected in at least one of the samples in at least one of the ionization modes. We used methods from [58] and the accompanying R package `imputeLCMD` to evaluate different imputation methods for imputing missing data, settling on the quantile regression imputation of left-censored (QRILC) data method. Imputed values theoretically represent values below the limit of detection rather than a complete absence of compounds. As expected, our imputed values always fell below the detected values for a given compound (Fig. S2). After imputation was performed, correlations between the replicates were high (Fig. S1B-C). Compounds that were detected in both ionization modes show a modest correlation within a sample (Fig. 1D). Distributions of normalized peak areas are similar across replicates and samples (Fig. S1C). This completed dataset was used for

further analysis and is available as supplementary table 1. Where appropriate, we show data from both ionization modes, treating the combination of a compound and the ionization mode it was detected in as a feature of the data and compare these across samples.

5.1.5 Theoretical distributions for parallel changes in metabolites

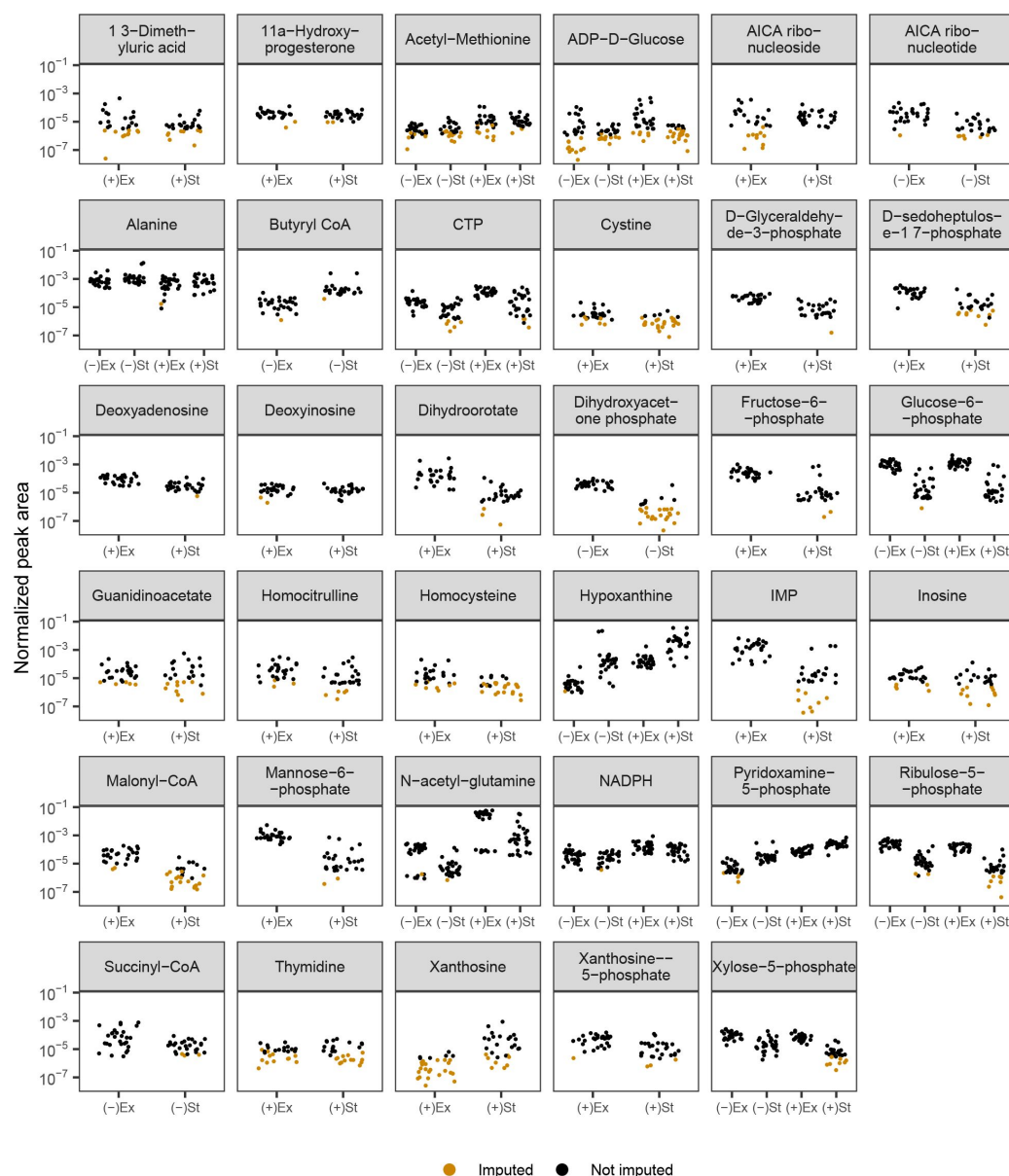
The code for this analysis can be found in the document titled `parallelism.Rmd`. We used the R package SINIB [33] to calculate theoretical probabilities of finding a shared metabolic change in a particular number of evolved lines. First, we designated metabolic features (the combination of compound and the ionization mode it was detected in) as significant if they experienced an $|\log_2(\text{fold-change})| \geq 1$. Then, we determined the probability of randomly picking a significantly altered metabolic feature in each evolved line in a manner specific to the growth phase and direction of change. We then parameterized the `dsinib` function with these probabilities, essentially asking what the chance of repeatedly finding the same change in an increasing number of evolved lines is. We used these probabilities to determine the total number of metabolic features ones might expect to find altered in the same direction. These numbers can be compared to the observed distribution, showing that more parallel changes are observed than expected.

6 Supplementary figures



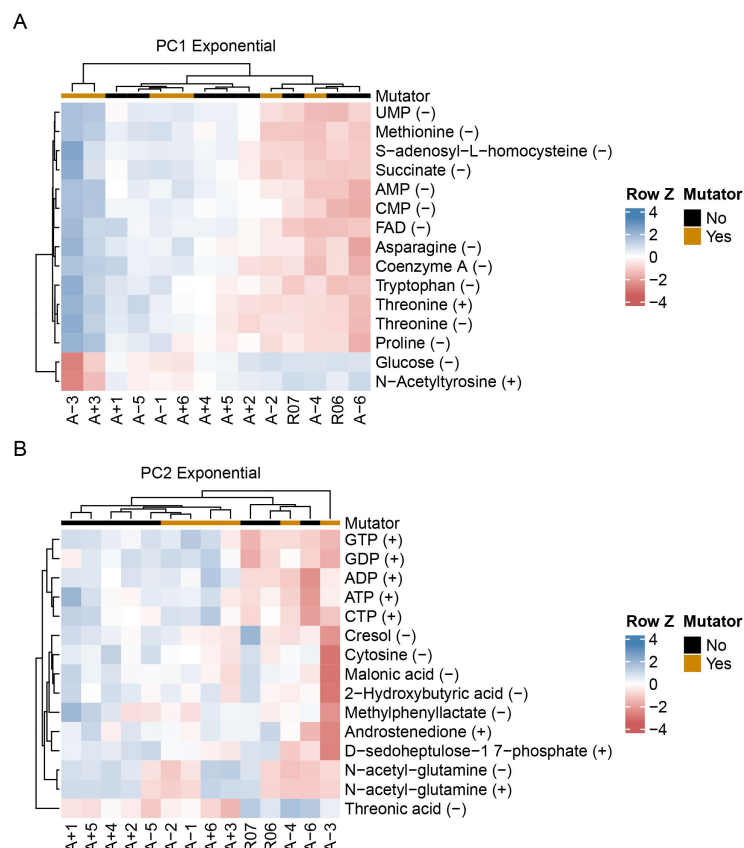
Supplementary Figure 1.

(A) Distributions of different pairwise correlations based on $\log_{10}(\text{normalized peak area})$, both (+) and (-) ionization mode data are considered. Data is from all samples with no averaging of replicates. Pairwise indicates all possible pairwise correlations. Replicates indicate comparisons of biological replicates. Intraphase and interphase are comparisons within or across growth phases, respectively. P-value indicates the result of a t-test testing if the within growth phase distribution is greater than the interphase distribution. **(B)** Correlations of compounds across ionization mode. Each point is the correlation of (+) and (-) ionization modes within a single evolved line. **(C)** Distributions of normalized peak areas are similar across replicates and samples. (+) and (-) indicate ionization mode and colors indicate replicates.



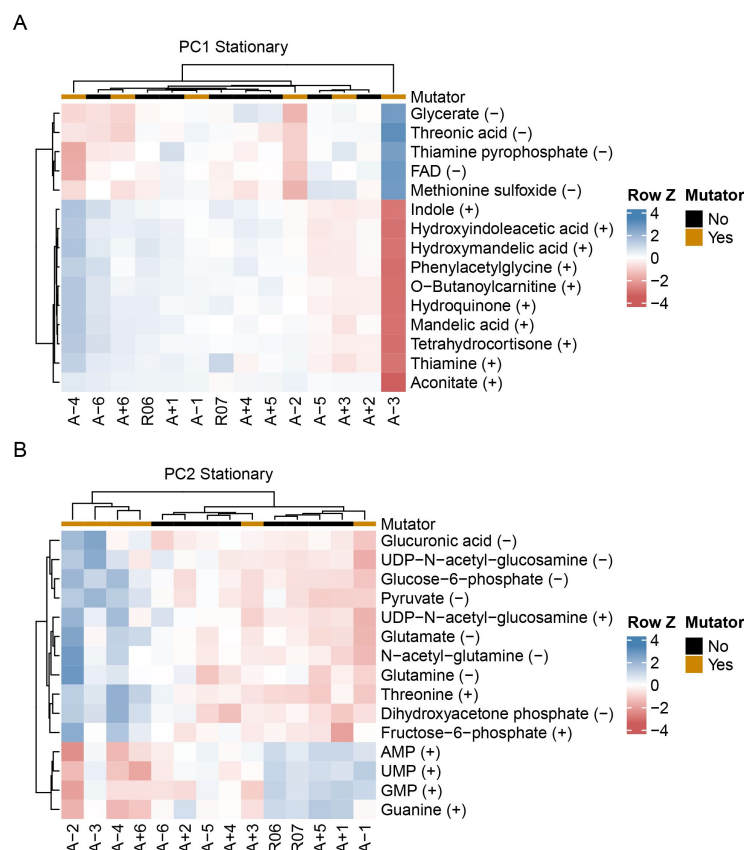
Supplementary Figure 2.

Distributions of peak areas for compounds whose values were imputed using a QRILC method (see [section 5.1.4](#) for a complete description). Imputed values are in orange. The combination of the growth phase (exponential, Ex; stationary, St) and the ionization mode (positive, (+); negative, (-)) is listed on the x-axis.



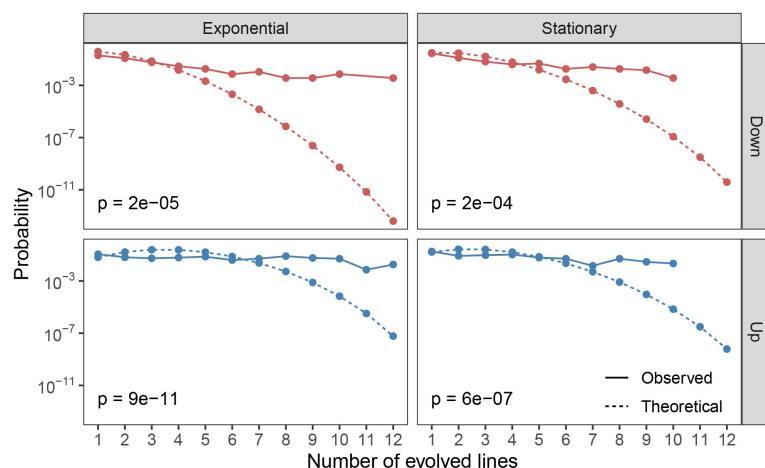
Supplementary Figure 3.

(A-B) The top 15 compounds contributing to PC1 and PC2 for the exponential phase metabolomes. The (+) or (-) next to a compound indicates the ionization mode of detection, not the charge of the molecule. Colors indicate the row-wise Z-scores based on normalized peak areas.



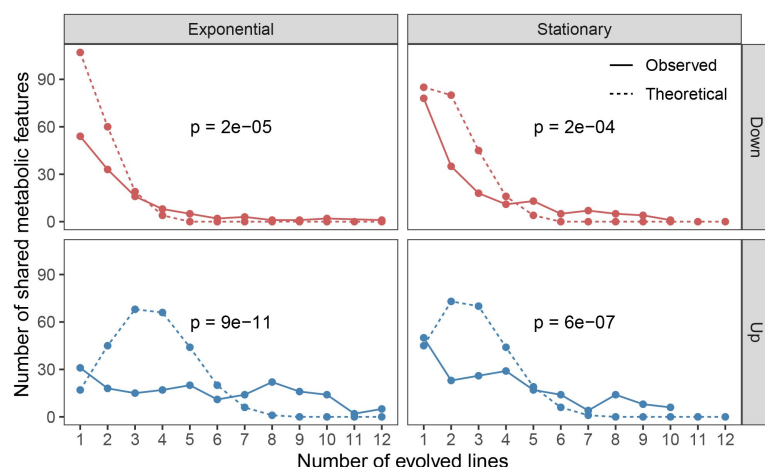
Supplementary Figure 4.

(A-B) The top 15 compounds contributing to PC1 and PC2 for the stationary phase metabolomes. The (+) or (-) next to a compound indicates the ionization mode of detection, not the charge of the molecule. Colors indicate the row-wise Z-scores based on normalized peak areas.



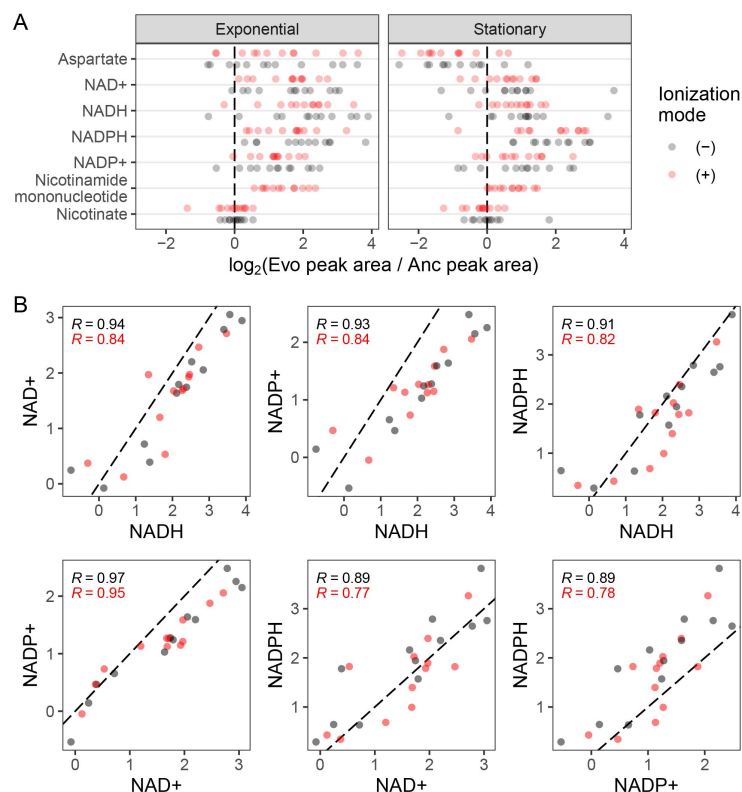
Supplementary Figure 5.

The theoretical and observed probabilities of finding features (the combination of metabolite and the ionization mode it was detected in) that are significantly altered ($|\log_2(\text{fold-change})| \geq 1$) in a given number of evolved lines (x-axis). Up and down refer to metabolites that are increased and decreased relative to the ancestral strain. Theoretical distributions were calculated using the Sum of Independent Non-Identical Binomial Random Variables (SINIB) method [33]. For more details on this method, see [section 5.1.5](#).



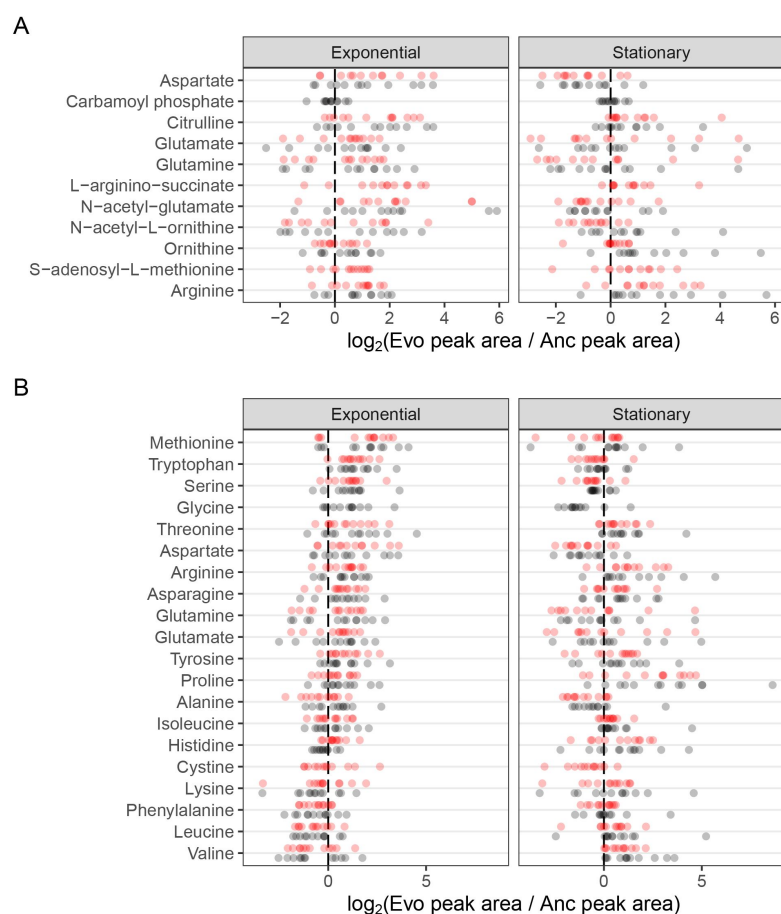
Supplementary Figure 6.

The theoretical and observed number of shared, significantly altered ($|\log_2(\text{fold-change})| \geq 1$) metabolic features (the combination of metabolite and the ionization mode it was detected in) in a given number of evolved lines (x-axis). P-values represent two-tailed t-tests between the observed and theoretical distributions of the number of shared features. The predicted number of shared metabolic features is calculated based on the theoretical probabilities in [Fig. S5](#). Up and down refer to metabolites that are increased and decreased relative to the ancestral strain.



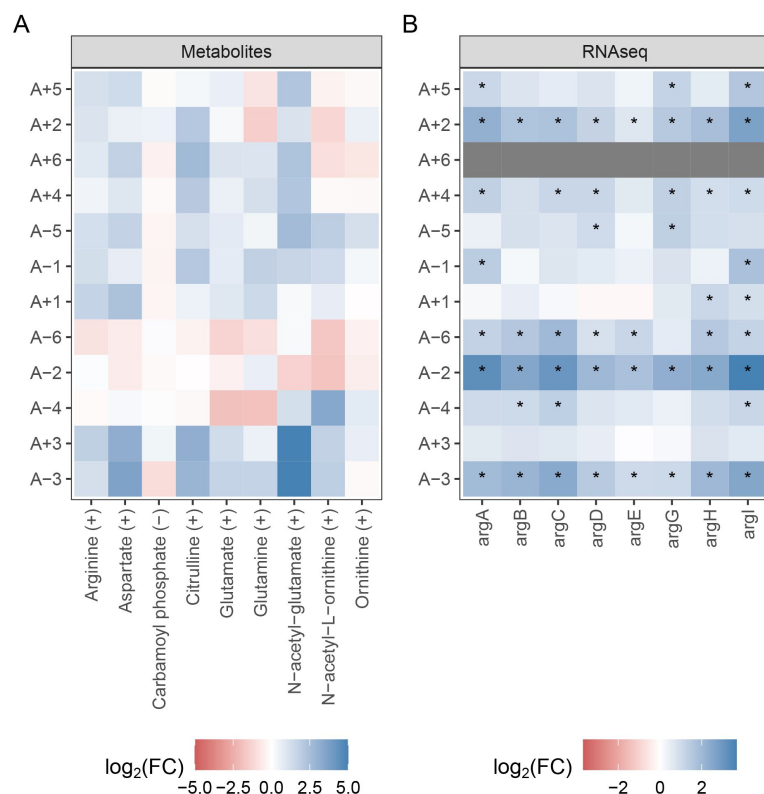
Supplementary Figure 7.

(A) Complete data for Fig. 2. The distribution of fold-changes relative to the ancestor for each compound in each ionization mode is shown. Fold-change is calculated after averaging replicates normalized peak areas. The ancestors are averaged together. **(B)** Correlations between the various NAD derived compounds. Each axis represents the exponential phase $\log_2(\text{fold-change})$ relative to the ancestor for that compound, and each point is an evolved line. The dotted line is the 1:1 line.



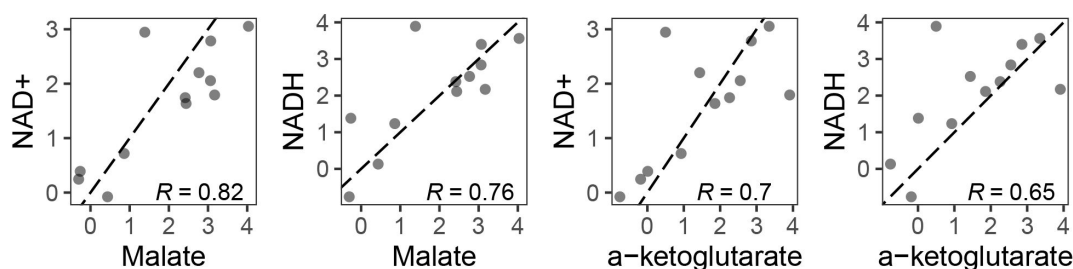
Supplementary Figure 8.

(A) Complete data for Fig. 3. The distribution of fold-changes relative to the ancestor for each compound in each ionization mode is shown. Compounds are ordered from top to bottom roughly as they occur in the pathway. **(B)** Fold-change values for all amino acids. Amino acids are ordered by median fold-change in the exponential phase across both ionization modes.



Supplementary Figure 9.

(A) Fold-changes for arginine-associated metabolites shown in a line specific manner. Compounds are ordered from top to bottom roughly as they occur in the pathway and lines are clustered based on fold-changes. Compounds are shown in positive ionization mode or in negative mode if they were not detected in positive mode. **(B)** Changes in gene expression for arginine-associated genes. RNAseq data is from [15], and asterisks indicate a statistically significant change in expression. A+6 did not have RNAseq data.



Supplementary Figure 10.

The relationship between fold-changes in malate and α -ketoglutarate and NAD(H) are correlated within an individual evolved line. Because malate and α -ketoglutarate were only detected in negative ionization mode, only data from this mode is shown. The dotted line is the 1:1 line.

References

- (1) Dobzhansky T. (1964) **Dobzhansky, T. *Am. Zool.* 1964, 4, 443–452. *Am. Zool* 4:443–452**
- (2) Harrison B. R. , Hoffman J. M. , Samuelson A. , Raftery D. , Promislow D. E. L. (2021) **Mol. Biol. Evol**
- (3) Santamaria G. , Liao C. , Lindberg C. , Chen Y. , Wang Z. , Rhee K. , Pinto F. R. , Yan J. , Xavier J. B. (2022) **Santamaria, G.; Liao, C.; Lindberg, C.; Chen, Y.; Wang, Z.; Rhee, K.; Pinto, F. R.; Yan, J.; Xavier, J. B. *Elife* 2022, 11. *Elife* 11**
- (4) Ibarra R. U. , Edwards J. S. , Palsson B. O. (2002) **Ibarra, R. U.; Edwards, J. S.; Palsson, B. O. *Nature* 2002, 420, 186–189. *Nature* 420:186–189**
- (5) Spencer C. C. , Tyerman J. , Bertrand M. , Doebeli M. (2008) **Spencer, C. C.; Tyerman, J.; Bertrand, M.; Doebeli, M. *Proc. Natl. Acad. Sci. U. S. A.* 2008, 105, 1585–1589. *Proc. Natl. Acad. Sci. U. S. A* 105:1585–1589**
- (6) Lenski R. E. , Rose M. R. , Simpson S. C. , Tadler S. C. (1991) **Lenski, R. E.; Rose, M. R.; Simpson, S. C.; Tadler, S. C. *Am. Nat.* 1991, 138, 1315–1341. *Am. Nat* 138:1315–1341**
- (7) Lenski R. E. , et al. (2015) **Lenski, R. E. et al. *Proc. Biol. Sci.* 2015, 282, 20152292. *Proc. Biol. Sci* 282**
- (8) Grant N. A. , Abdel Magid A. , Franklin J. , Dufour Y. , Lenski R. E. (2021) **Grant, N. A.; Abdel Magid, A.; Franklin, J.; Dufour, Y.; Lenski, R. E. *J. Bacteriol.* 2021, 203. *J. Bacteriol* 203**
- (9) Philippe N. , Pelosi L. , Lenski R. E. , Schneider D. (2009) **Philippe, N.; Pelosi, L.; Lenski, R. E.; Schneider, D. *J. Bacteriol.* 2009, 191, 909–921. *J. Bacteriol* 191:909–921**
- (10) Tenaillon O. , Barrick J. E. , Ribeck N. , Deatherage D. E. , Blanchard J. L. , Dasgupta A. , Wu G. C. , Wielgoss S. , Cruveiller S. , Médigue C. , Schneider D. , Lenski R. E. (2016) **Tenaillon, O.; Barrick, J. E.; Ribeck, N.; Deatherage, D. E.; Blanchard, J. L.; Dasgupta, A.; Wu, G. C.; Wielgoss, S.; Cruveiller, S.; Médigue, C.; Schneider, D.; Lenski, R. E. *Nature* 2016, 536, 165–170. *Nature* 536:165–170**
- (11) Tenaillon O. , Rodríguez-Verdugo A. , Gaut R. L. , McDonald P. , Bennett A. F. , Long A. D. , Gaut B. S. (2012) **Tenaillon, O.; Rodríguez-Verdugo, A.; Gaut, R. L.; McDonald, P.; Bennett, A. F.; Long, A. D.; Gaut, B. S. *Science* 2012, 335, 457–461. *Science* 335:457–461**
- (12) Good B. H. , McDonald M. J. , Barrick J. E. , Lenski R. E. , Desai M. M. (2017) **Good, B. H.; McDonald, M. J.; Barrick, J. E.; Lenski, R. E.; Desai, M. M. *Nature* 2017, 551, 45–50. *Nature* 551:45–50**
- (13) Limdi A. , Owen S. V. , Herren C. , Lenski R. E. , Baym M. (2022) **Parallel changes in gene essentiality over 50,000 generations of evolution., en**

- (14) Cooper T. F. , Rozen D. E. , Lenski R. E. (2003) **Cooper, T. F.; Rozen, D. E.; Lenski, R. E.** *Proc. Natl. Acad. Sci. U. S. A.* **2003**, **100**, **1072–1077**. *Proc. Natl. Acad. Sci. U. S. A* **100**:1072–1077
- (15) Favate J. S. , Liang S. , Cope A. L. , Yadavalli S. S. , Shah P. (2022) **Favate, J. S.; Liang, S.; Cope, A. L.; Yadavalli, S. S.; Shah, P.** *Elife* **2022**, **11**. *Elife* **11**
- (16) Blount Z. D. , Barrick J. E. , Davidson C. J. , Lenski R. E. (2012) **Blount, Z. D.; Barrick, J. E.; Davidson, C. J.; Lenski, R. E.** *Nature* **2012**, **489**, **513–518**. *Nature* **489**:513–518
- (17) Quandt E. M. , Gollihar J. , Blount Z. D. , Ellington A. D. , Georgiou G. , Barrick J. E. (2015) **Quandt, E. M.; Gollihar, J.; Blount, Z. D.; Ellington, A. D.; Georgiou, G.; Barrick, J. E.** *Elife* **2015**, **4**. *Elife* **4**
- (18) Lenski R. E. (2017) **Lenski, R. E.** *ISME J.* **2017**, **11**, **2181–2194**. *ISME J* **11**:2181–2194
- (19) Pluacain J. , Hindré T. , Le Gac M. , Tenaillon O. , Cruveiller S. , Médigue C. , Leiby N. , Harcombe W. R. , Marx C. J. , Lenski R. E. , Schneider D. (2014) **Pluacain, J.; Hindré, T.; Le Gac, M.; Tenaillon, O.; Cruveiller, S.; Médigue, C.; Leiby, N.; Harcombe, W. R.; Marx, C. J.; Lenski, R. E.; Schneider, D.** *Science* **2014**, **343**, **1366–1369**. *Science* **343**:1366–1369
- (20) Rozen D. E. , Lenski R. E. (2000) **Rozen, D. E.; Lenski, R. E.** *Am. Nat.* **2000**, **155**, **24–35**. *Am. Nat* **155**:24–35
- (21) Cooper V. S. , Schneider D. , Blot M. , Lenski R. E. (2001) **Cooper, V. S.; Schneider, D.; Blot, M.; Lenski, R. E.** *J. Bacteriol.* **2001**, **183**, **2834–2841**. *J. Bacteriol* **183**:2834–2841
- (22) Meyer J. R. , Agrawal A. A. , Quick R. T. , Dobias D. T. , Schneider D. , Lenski R. E. (2010) **Meyer, J. R.; Agrawal, A. A.; Quick, R. T.; Dobias, D. T.; Schneider, D.; Lenski, R. E.** *Evolution* **2010**, **64**, **3024–3034**. *Evolution* **64**:3024–3034
- (23) Pelosi L. , Kühn L. , Guetta D. , Garin J. , Geiselmann J. , Lenski R. E. , Schneider D. (2006) **Pelosi, L.; Kühn, L.; Guetta, D.; Garin, J.; Geiselmann, J.; Lenski, R. E.; Schneider, D.** *Genetics* **2006**, **173**, **1851–1869**. *Genetics* **173**:1851–1869
- (24) Travisano M. , Vasi F. , Lenski R. E. (1995) **Travisano, M.; Vasi, F.; Lenski, R. E.** *Evolution* **1995**, **49**, **189–200**. *Evolution* **49**:189–200
- (25) Leiby N. , Marx C. J. (2014) **Leiby, N.; Marx, C. J.** *PLoS Biol.* **2014**, **12**, **e1001789**. *PLoS Biol* **12**
- (26) Harcombe W. R. , Delaney N. F. , Leiby N. , Klitgord N. , Marx C. J. (2013) **Harcombe, W. R.; Delaney, N. F.; Leiby, N.; Klitgord, N.; Marx, C. J.** *PLoS Comput. Biol.* **2013**, **9**, **e1003091**. *PLoS Comput. Biol* **9**
- (27) Blount Z. D. , Borland C. Z. , Lenski R. E. (2008) **Blount, Z. D.; Borland, C. Z.; Lenski, R. E.** *Proc. Natl. Acad. Sci. U. S. A.* **2008**, **105**, **7899–7906**. *Proc. Natl. Acad. Sci. U. S. A* **105**:7899–7906

- (28) Jaishankar J. , Srivastava P. (2017) **Jaishankar, J.; Srivastava, P. *Front. Microbiol.* 2017, 8, 2000. *Front. Microbiol* 8**
- (29) Pletnev P. , Osterman I. , Sergiev P. , Bogdanov A. , Dontsova O. (2015) **Pletnev, P.; Osterman, I.; Sergiev, P.; Bogdanov, A.; Dontsova, O. *Acta Naturae* 2015, 7, 22–33. *Acta Naturae* 7:22–33**
- (30) Navarro Llorens J. , Tormo A. , Martínez-García E. (2010) **Navarro Llorens, J. M.; Tormo, A.; Martínez-García, E. *FEMS Microbiol. Rev.* 2010, 34, 476–495. *FEMS Microbiol. Rev* 34:476–495**
- (31) Turner C. B. , Wade B. D. , Meyer J. R. , Sommerfeld B. A. , Lenski R. E. (2017) **Turner, C. B.; Wade, B. D.; Meyer, J. R.; Sommerfeld, B. A.; Lenski, R. E. *R Soc Open Sci* 2017, 4, 170497. *R Soc Open Sci* 4**
- (32) Wiser M. J. , Ribeck N. , Lenski R. E. (2013) **Wiser, M. J.; Ribeck, N.; Lenski, R. E. *Science* 2013, 342, 1364–1367. *Science* 342:1364–1367**
- (33) Liu B. , Quertermous T. (2018) **Liu, B.; Quertermous, T. *R J* 2018, 10, 472. *R J* 10**
- (34) Woods R. , Schneider D. , Winkworth C. L. , Riley M. A. , Lenski R. E. (2006) **Woods, R.; Schneider, D.; Winkworth, C. L.; Riley, M. A.; Lenski, R. E. *Proc. Natl. Acad. Sci. U. S. A.* 2006, 103, 9107–9112. *Proc. Natl. Acad. Sci. U. S. A* 103:9107–9112**
- (35) Begley T. P. , Kinsland C. , Mehl R. A. , Osterman A. , Dorrestein P. (2001) **In *Vitamins & Hormones* :103–119**
- (36) Osterman A. (2009) **Osterman, A. *EcoSal Plus* 2009, 3. *EcoSal Plus* 3**
- (37) Keseler I. M. , Collado-Vides J. , Gama-Castro S. , Ingraham J. , Paley S. , Paulsen I. T. , Peralta-Gil M. , Karp P. D. (2005) **Keseler, I. M.; Collado-Vides, J.; Gama-Castro, S.; Ingraham, J.; Paley, S.; Paulsen, I. T.; Peralta-Gil, M.; Karp, P. D. *Nucleic Acids Res.* 2005, 33, D334–7. *Nucleic Acids Res* 33:D334–7**
- (38) Grose J. H. , Bergthorsson U. , Roth J. R. (2005) **Grose, J. H.; Bergthorsson, U.; Roth, J. R. *J. Bacteriol.* 2005, 187, 2774–2782. *J. Bacteriol* 187:2774–2782**
- (39) Grose J. H. , Joss L. , Velick S. F. , Roth J. R. (2006) **Grose, J. H.; Joss, L.; Velick, S. F.; Roth, J. R. *Proc. Natl. Acad. Sci. U. S. A.* 2006, 103, 7601–7606. *Proc. Natl. Acad. Sci. U. S. A* 103:7601–7606**
- (40) Cantó C. , Menzies K. J. , Auwerx J. (2015) **Cantó, C.; Menzies, K. J.; Auwerx, J. *Cell Metab.* 2015, 22, 31–53. *Cell Metab* 22:31–53**
- (41) Kawai S. , Mori S. , Mukai T. , Hashimoto W. , Murata K. (2001) **Kawai, S.; Mori, S.; Mukai, T.; Hashimoto, W.; Murata, K. *Eur. J. Biochem.* 2001, 268, 4359–4365. *Eur. J. Biochem* 268:4359–4365**

- (42) Tian G. , Maas W. K. (1994) **Tian, G.; Maas, W. K. Mol. Microbiol. 1994, 13, 599–608. Mol. Microbiol 13:599–608**
- (43) Charlier D. , Glansdorff N. (2004) **Charlier, D.; Glansdorff, N. EcoSal Plus 2004, 1. EcoSal Plus 1**
- (44) Reitzer L. (2005) **Reitzer, L. EcoSal Plus 2005, 1. EcoSal Plus 1**
- (45) Cioni M. , Pinzauti G. , Vanni P. (1981) **Cioni, M.; Pinzauti, G.; Vanni, P. Comparative Biochemistry and Physiology Part B: Com-parative Biochemistry 1981, 70, 1–26. Comparative Biochemistry and Physiology Part B: Com-parative Biochemistry 70:1–26**
- (46) Philippe N. , Crozat E. , Lenski R. E. , Schneider D. (2007) **Philippe, N.; Crozat, E.; Lenski, R. E.; Schneider, D. Bioessays 2007, 29, 846–860. Bioessays 29:846–860**
- (47) Warburg O. (1956) **Warburg, O. Science 1956, 123, 309–314. Science 123:309–314**
- (48) Enjalbert B. , Millard P. , Dinclaux M. , Portais J.-C. , Létisse F. (2017) **Enjalbert, B.; Millard, P.; Dinclaux, M.; Portais, J.-C.; Létisse, F. Sci. Rep. 2017, 7, 42135. Sci. Rep 7**
- (49) Miyazawa K. , Noguchi T. (2001) **Miyazawa, K.; Noguchi, T. J. Toxicol. Toxin Rev. 2001, 20, 11–33. J. Toxicol. Toxin Rev 20:11–33**
- (50) Hefetz A. , Blum M. S. (1978) **Hefetz, A.; Blum, M. S. Biochim. Biophys. Acta 1978, 543, 484–496. Biochim. Biophys. Acta 543:484–496**
- (51) Chevette M. G. , Gutiérrez-García K. , Selem-Mojica N. , Aguilar-Martínez C. , Yañez-Olvera A. , Ramos-Aboites H. E. , Hoskisson P. A. , Barona-Gómez F. (2020) **Chevette, M. G.; Gutiérrez-García, K.; Selem-Mojica, N.; Aguilar-Martínez, C.; Yañez-Olvera, A.; Ramos-Aboites, H. E.; Hoskisson, P. A.; Barona-Gómez, F. Nat. Prod. Rep. 2020, 37, 566–599. Nat. Prod. Rep 37:566–599**
- (52) Sajed T. , Marcu A. , Ramirez M. , Pon A. , Guo A. C. , Knox C. , Wilson M. , Grant J. R. , Djoumbou Y. , Wishart D. S. (2016) **Sajed, T.; Marcu, A.; Ramirez, M.; Pon, A.; Guo, A. C.; Knox, C.; Wilson, M.; Grant, J. R.; Djoumbou, Y.; Wishart, D. S. Nucleic Acids Res. 2016, 44, D495–501. Nucleic Acids Res 44:D495–501**
- (53) Katz S. , Avrani S. , Yavneh M. , Hilau S. , Gross J. , Hershberg R. (2021) **Katz, S.; Avrani, S.; Yavneh, M.; Hilau, S.; Gross, J.; Hershberg, R. Mol. Biol. Evol. 2021, 38, 2778–2790. Mol. Biol. Evol 38:2778–2790**
- (54) Ratib N. R. , Seidl F. , Ehrenreich I. M. , Finkel S. E. (2021) **Ratib, N. R.; Seidl, F.; Ehrenreich, I. M.; Finkel, S. E. MBio 2021, 12. MBio 12**
- (55) Su X. , Chiles E. , Maimouni S. , Wondisford F. E. , Zong W.-X. , Song C. (2020) **Su, X.; Chiles, E.; Maimouni, S.; Wondisford, F. E.; Zong, W.-X.; Song, C. Anal. Chem. 2020, 92, 4829–4837. Anal. Chem 92:4829–4837**

(56) R Core Team R (2022) **A Language and Environment for Statistical Computing**

(57) Wickham H. , et al. (2019) **Wickham, H. et al. JOSS 2019, 4, 1686. JOSS 4**

(58) Wei R. , Wang J. , Su M. , Jia E. , Chen S. , Chen T. , Ni Y. (2018) **Wei, R.; Wang, J.; Su, M.; Jia, E.; Chen, S.; Chen, T.; Ni, Y. Sci. Rep. 2018, 8, 663. Sci. Rep 8**

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Reviewer #1 (Public Review):

Summary:

Favate et al. measure the relative levels of metabolites in 12 *Escherichia coli* strains isolated from different replicate populations after 50,000 generations of the Lenski long-term laboratory evolution experiment. They use untargeted LC/MS methods that include standards and report both positive and negative ionization mode measurements. They initially use principal component analysis (PCA) to broadly compare how the metabolomes of these strains are similar and different. Then, they describe several instances where the changes in metabolite abundance they see in specific pathways correlate with mutations that lead to changes in the expression of genes that encode enzymes in those pathways.

Strengths:

The statistical analyses and presentation of the high-throughput data are excellent. The most compelling results are communicated in wonderful figures that integrate their measurements of metabolite levels in this study with results from a prior study they conducted looking at changes in gene expression levels in the same bacterial strains. These sections include the ones describing large increases in NAD(P) pools due to mutations in *nadR*, changes in the levels of arginine and related compounds due to mutations in *argR*, and changes in metabolites from glycolysis and the TCA cycle related to *iclR* and *arcB*.

Weaknesses:

After addressing prior reviews, the main remaining weaknesses of the study are limitations inherent to the metabolomics approach that are noted by the authors. Namely, that it gives a static and incomplete picture of cellular metabolism, lacking any information about flux and missing measurements for many metabolites. Additional biochemical and genetic experiments will be necessary to fully test the hypotheses suggested by the metabolomics data.

Impact and Significance:

While there has been past speculation about the effects of LTEE mutations on metabolism, this study measures changes in the levels of metabolites in related metabolic pathways for the first time. Therefore, it provides useful information about how metabolism evolves, in general, and will also be a useful resource those studying other aspects of the LTEE related to metabolism, such as contingency in the evolution of citrate utilization.

Reviewer #2 (Public Review):

This preprint presents a compelling study examining the relationship between genotypic changes and phenotypic traits in bacteria over an extended period using the Long-Term Evolution Experiment (LTEE) as a model. The primary advances in methodology include employing high-resolution mass spectrometry for comprehensive metabolic profiling and combining it with previous gene expression and DNA sequencing datasets. This approach provides insight into how specific genetic mutations can alter metabolic pathways over 50,000 generations, enabling a deeper understanding of how genetic changes lead to observed differences in evolved bacterial strains. The findings reveal that evolved bacteria possess more diverse metabolic profiles compared to their ancestors, suggesting that these populations have uniquely adapted to their environment. The work also attempts to uncover

the molecular basis for this adaptive evolution, demonstrating how specific genetic changes have influenced the bacteria's metabolic pathways.

Overall, this is a significant and well-executed research study. It offers new insights into the complex relationship between genetic changes and observable traits in evolving populations and utilizes metabolomics in the LTEE, a novel approach in combination with RNA-seq and mutation datasets.

Author Response

The following is the authors' response to the original reviews.

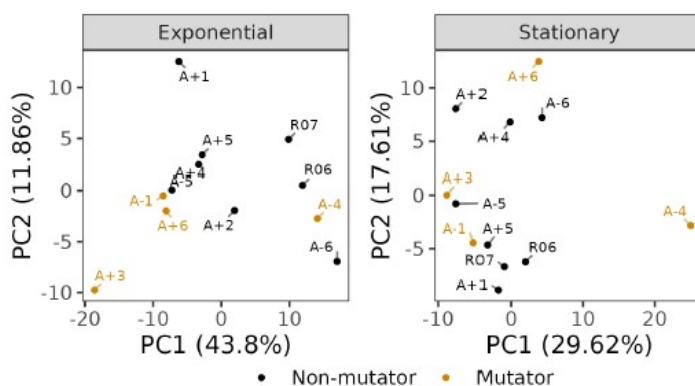
Reviewer #1 (Public Review):

[...] Weaknesses

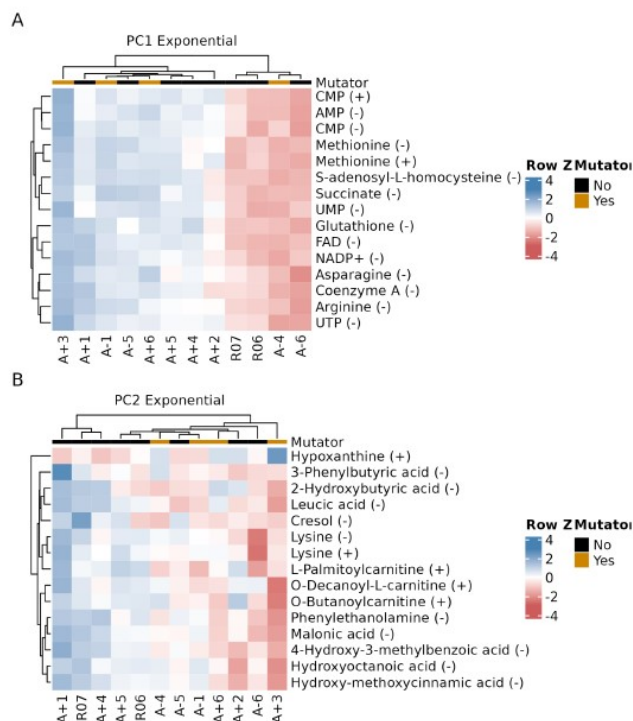
Showing that A-2 and especially A-3 are outliers in the PCA analysis is useful, but it may be hiding other interesting signals in the data. The other strains are remarkably colinear on these plots, hinting that if the outliers were removed, one main component would emerge along which they are situated. It also seems possible that this additional analysis step would allow the second dimension to better differentiate them in a way that is interesting with respect to their mutator status or mutations in key metabolic or regulatory genes.

We thank the reviewer for their positive comments and their constructive feedback on the manuscript. Following reviewer's recommendation, we performed the PCA analysis on metabolism data after removing A-2 and A-3 data. We have detailed those results below. Consistent with a similar analysis performed on RNA-seq datasets in our previous publication, we find that removing these outliers has only a modest effect on separating mutators from non-mutators. We find that, while the new PC2 separates most mutators from the non-mutators, the separation is rather weak. Moreover, we do not see a similar distinction when looking at metabolic data in the Stationary phase. In the interest of improving the readability of the manuscript, we recommend not including these analysis in the final manuscript. We have presented the data for the reviewer's benefit in Author response image 1, 2 and 3.

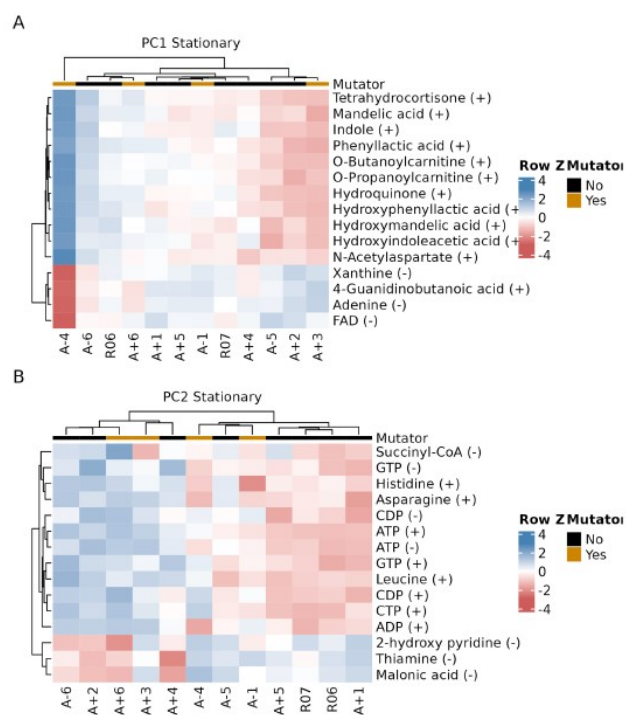
Author response image 1.



Author response image 2.



Author response image 3.



There is a missed opportunity to connect some key results to what is known about LTEE mutations that reduce the activity of pykF (pyruvate kinase I). This gene is mutated in all 12 LTEE populations, and often these mutations are frameshifts or transposon insertions that should completely knock out its activity. At first glance, inactivating an enzyme for a step in glycolysis does not make sense when the nutrient source in the growth medium is glucose, even though PykF is only one of two isozymes E. coli encodes for this reaction. There has been speculation that inactivating pykF increases the concentration of phosphoenolpyruvate (PEP) in cells and that this can lead to increased rates of glucose import because PEP is used by the phosphotransferase system of E. coli to import glucose (see <https://doi.org/10.1002/bies.20629>). The current study has confirmed the higher PEP levels, which is consistent with this model.

We thank the reviewer for pointing out this missed opportunity. We have expanded the discussion around the role of pykF mutations and the elevated concentrations of PEP observed in our data in section 3.4.

In the introduction, the papers cited to show the importance of changes in metabolism for adaptation do not seem to fit the focus of this study very well. They stress production of toxins and secondary metabolites, which do not seem to be mechanisms that are at work in the LTEE. I can think of two areas of background that would be more relevant: (1) studies of how bacterial metabolism evolves in adaptive laboratory evolution (ALE) experiments to optimize metabolic fluxes toward biomass production (for example, <https://doi.org/10.1038/nature01149>), and (2) discussions of how cross-feeding, metabolic niche specialization, and metabolic interdependence evolve in microbial communities, including in other evolution experiments (for example, <https://doi.org/10.1073/pnas.0708504105> and <https://doi.org/10.1126/mBio.00036-12>).

We thank the reviewer for pointing out missed citations in our introduction. We agree that these papers are relevant to the topic and have added their citations. Additionally, following the suggestion of another reviewer, we have reorganized the introduction so that the concept of the role of metabolism in evolution is presented first and the LTEE second.

Reviewer #2 (Public Review):

[...] Overall, this is a significant and well-executed research study. It offers new insights into the complex relationship between genetic changes and observable traits in evolving populations and utilizes metabolomics in the LTEE, a novel approach in combination with RNA-seq and mutation datasets.

However, the paper's overall clarity is lacking. It is spread too thin and covers many topics without a clear focus. I strongly recommend a substantial rewrite of the manuscript, emphasizing structure and readability. The science is well executed, but the current writing does not do it justice.

We thank the reviewer for their positive comments and their constructive feedback on the lack of clarity in writing. Following the reviewer's suggestions, we have rewritten parts of the manuscript and reorganized a few sections to improve readability. We hope the revised manuscript is significantly improved.

Recommendations for the authors

Reviewer #1 (Recommendations For The Authors):

1. *Title and Abstract: Add the study organism to the abstract, and probably also the title. Currently, E. coli is not mentioned in either! I'm also not sure that the LTEE is a sufficiently well-known acronym to abbreviate this in the title.*

We have revised the title of the manuscript and now spell out LTEE and included E. coli in the title and the abstract.

1. *Abstract: I would switch the usage of metabolome to metabolism in a few more places. For example, "changes in its metabolism", "networked and convoluted nature of metabolism". The metabolome, the concentrations of all metabolites, is what is being measured, but I think of this as a phenotypic readout of how metabolism evolving.*

We have changed “metabolome” to “metabolism” in cases where we refer to what is evolving and use “metabolome” when we refer to what is being measured.

1. *Line 16: Technically, the 12 LTEE populations were not initially identical. The Ara- differed from the Ara+ ancestors by one intentional mutation and one unintentional mutation that was not discovered until whole genomes were sequenced. I would rephrase this to "where 12 replicate populations of E. coli are propagated" or something similar so that it can be correct without needing to describe this unnecessary detail.*

The line has been rephrased as suggested.

1. *General Note: The text refers to populations as Ara-3 but the figures use A-3. I'd suggest going with A-3 and similar throughout for consistency.*

Instances of Ara have been changed to A+/-, and a sentence specifying as such has been added to the intro to make mention of this.

1. *Lines 43-44, 97-98. My understanding is that both S and L ecotypes in A-2 can use both glucose and acetate, but that the differentiation is related to their specialization that leads to each one being better on one or the other nutrient. The descriptions make it sound like each grows at a different time. Also, by definition, cells are not growing during "stationary phase". The change from glucose utilization (and acetate secretion) to acetate utilization during one cycle of growth is better described as a diauxic shift.*

We have reworded this part to remove mention of “growth” during stationary phase and changed the wording such that it no longer sounds like they grow at different times.

1. *Line 54: The statement "provide the ability to test hypotheses from previous data" is vague. Either provide an example or delete.*

We have removed this sentence as suggested.

1. *Lines 71-72: The terms "interphase" and "intrapphase" sound too much like parts of the cell cycle. I'd suggest describing the comparisons as between and within growth phases.*

The use of intra and interphase have been changed as suggested.

1. Line 79: The citrate is presumably still a chelating agent, so change phrasing to "Citrate is present in the medium because it was originally added as a chelating agent" or something similar.

This sentence has been rewritten as suggested.

1. Line 83: Write out "mutation accumulations" so it is easier to understand as "the number of mutations that have accumulated".

The phrase has been changed as suggested.

1. Line 116: It's unclear whether the abundances of metabolites are "strategies of survival" in stationary phase. An equally valid explanation is that there is less selection on the metabolome to have a specific composition during stationary phase to have high fitness.

We have added a line about the possibility for alternative hypotheses.

1. Figure 1: There seems to be some information missing from the legend. What are R06 and R07 in Panels A and B? Is panel D exponential phase and panel E stationary phase?

This information was inadvertently missing from the caption and has been added.

1. Figures 2 and 3: Gene names should be in italics. To me, the gray for deleted genes is hard to tell apart from the blue/red. Perhaps you could put a little X in these boxes instead? I think that having a little triangle pointing from each gene or metabolite name its corresponding abundance panel would help the reader track which information goes with which features. In Fig. 3 the placement of L-aspartate is a bit awkward. I'd suggest moving it down so the dashed line does not have to go through the abundance panel.

These figures have been edited to include small triangles that link a gene or metabolite and its heatmap. Additionally, an X has been added where genes have suffered inactivating mutations and the placement of some elements has been moved to improve overall clarity.

1. Lines 183-185: It would be easier to see and judge the consistency of these *argR* related relationships if a correlation graph of some kind was shown, probably as a supplemental figure. This plot could, for example, have genes/metabolites across the x-axis and fold-change on the y-axis with lines connecting points corresponding to each of the twelve populations across these categories (like Fig S8 but with lines added). Alternatively, it could be a heat map with the populations across one axis and the genes/metabolites across the other axis (like Fig S3).

We have added a supplementary figure consisting of heatmaps showing the consistency of these changes within an evolved line. It is now figure S9.

1. Line 195: I think adding a sentence elaborating on what exactly mutation accumulation means in this context would be helpful to readers.

We have attempted to clarify the meaning of this by specifically stating that it is due to the accumulation of deleterious mutations.

1. Line 293: Is standard LTEE medium DM25? These omics experiments with the LTEE sometimes use similar media with different glucose concentrations, and this is a very important detail to precisely specify.

We reference "standard" LTEE medium in the methods section and have additionally specified the amount of sugar to make it clear that we are not supplementing the media with additional sugar.

1. Figure S8B. Is "cystine" used instead of "cysteine" on purpose here since the compound is oxidized in the metabolomics treatment?

The use of cystine is intentional, we detect the oxidized compound.

Reviewer #2 (Recommendations For The Authors):

Title:

The abbreviation "LTEE" should not be in the title. Most readers will not recognize what it means. Instead, either the full name of the experiment, "Long-Term Evolution Experiment with E. coli," should be used, or the title should be rephrased to "Linking genotypic and phenotypic changes during a long-term evolution experiment using metabolomics."

We have spelled out LTEE and included E. coli in the title.

Abstract:

Sentence 1: Consider softening the statement: "Do changes in an organism's environment, genome, or gene expression patterns often lead to changes in its metabolome?"

We have rephrased this sentence to "Changes in an organism's environment, genome, or gene expression patterns can lead to changes in its metabolism".

Sentence 4: Use a hyphen for "Long-Term."

This addition has been made.

Sentence 4: Replace "transduce" with a more appropriate term: "...how the effects of mutations can be distributed through a cellular network to eventually affect metabolism and fitness."

We have rewritten this sentence as "to understand how mutations can eventually affect metabolism and perhaps fitness".

Sentence 5: Clarify the use of "both" to refer to the ancestor of the LTEE and its descendant populations as two classes.

We have reworded this sentence so it's clear that the ancestors and evolved lines are two separate classes "We used mass-spectrometry to broadly survey the metabolomes of the ancestral strains and all 12 evolved lines...".

Sentence 6: Reverse the order for better emphasis: "Our work provides a better understanding of how mutations might affect fitness through the metabolome in the LTEE, and thus provides a major step in developing a complete genotype-phenotype map for this experimental system."

We have rearranged this sentence per the reviewers suggestion.

Introduction:

Revise the introduction for clarity, readability, and logical narrative progression. Start with the second paragraph to set up the basic scientific principles being studied and then transition to describing the LTEE as a model system to examine those principles.

The introduction has been rearranged and reworded in parts to increase clarity.

Sentence 1: Revise for clarity: "The Long-Term Evolution Experiment (LTEE) has studied 12 initially identical populations of Escherichia coli as they have evolved in a carbon-limited, minimal glucose medium under a daily serial transfer regime."

Sentence 2: Suggestion: "Begun in 1988, the LTEE populations have evolved for more than 75,000 generations, making it the longest-running experiment of its kind."

Paragraph 2, sentence 2: Italicize "Drosophila."

Paragraph 3, sentence 2: Make an important distinction: "Ara-3 is unique in that it evolved the ability to grow aerobically on citrate."

Paragraph 3, sentence 4: Introduce the IS-mediated loss of the rbs operon in the LTEE as if it has not been described elsewhere.

These suggestions have been incorporated into the manuscript.

Results:

Section 3.1: The use of samples from hours 2 and 24 to represent exponential and stationary phase may present some issues. For instance, capturing Ara-3 during its exponential growth on glucose, but not citrate, at hour 2. Furthermore, except for Ara-3, the LTEE populations reach stationary phase after approximately 4 hours, and there could be significant differences between early, mid, and late stationary phase. This possibility should be acknowledged, and future follow-up work should consider exploring these differences.

We have added sentences in the first paragraph of the results section to include these details. We have also added a short paragraph to the conclusions suggesting additional studies of stationary phase, citing work on evolution of E. coli during long term stationary phase.

Paragraph 3: While Turner et al. 2017 is an essential reference regarding resource use differences between Ara-3 and other LTEE populations, it would be more suitable to reference Blount et al. 2012 for the mutations that enabled access to citrate. Also, it is important to note that the difference lies in the ability to grow aerobically on citrate, rather than the ability to metabolize it.

This citation has been added.

Paragraph 4: As mentioned elsewhere, most LTEE populations exhibit balanced polymorphisms. Therefore, it is more appropriate to state that Ara-2 is the best-understood example of long-term diversity. It is likely that there are important metabolic differences between co-existing lineages in other LTEE populations.

We now refer to Ara-2 as being the best-understood example of long term diversity..

Paragraph 5: The first sentence of this paragraph should likely end with "levels."

The word "levels" was added to the end of this sentence.

Figure 3: It is preferable to refer to the "Superpathway of arginine and polyamine biosynthesis," citing EcoCyc as a reference, rather than a descriptor.

This has been changed to a reference.

Section 3.3, Paragraph 3: While higher intracellular amino acid abundances may facilitate higher translation rates and faster growth, the higher abundances themselves do not evaluate the hypothesis. To evaluate the hypothesis, it is necessary to demonstrate that higher abundances are associated with higher translation or growth rates. Therefore, the final sentence of this paragraph is not meaningful.

We have reworded this sentence to say that it's not possible to tell what the additional amino acids are being used for given only this data and that additional experiments are needed to confirm this hypothesis.

Section 3.4: The first paragraph of this section misstates how evolution works. The low level of glucose in the LTEE does not drive innovation; instead, innovation occurs at random through the introduction of variation by mutation. Although the existence of the citrate resource acts as a reward that selects for variation that provides access to it, it is essential to remember that evolution is blind to such a reward. Moreover, regarding the evolution of the Cit⁺ trait, it is incorrect to assert that low glucose contributed to its evolution. As shown by Quandt et al. (2015), it seems probable that Cit⁺ evolution was potentiated by adaptation to specialization on acetate, which is produced by overflow metabolism resulting from rapid growth on glucose. This rapid growth only occurs when glucose is relatively abundant. The level of glucose seems low to us because it is low relative to traditional levels in bacteriological media, but not to the bacteria.

We agree that this is a semantical, but important distinction. We have reworded this part as to not suggest that evolution has any forward thinking properties and is indeed blind to any rewards that might occur as the result of adaptation.

In general, all instances of "utilize" and its cognates should be replaced with "use" and its cognates.

Instances of "utilize" have been changed to use and its cognates.

There is some uncertainty about the expectation of ramping up the TCA cycle in the LTEE. Overflow metabolism and acetate production appear to be prevalent in the LTEE, suggesting that many lineages only partially oxidize carbon derived from glucose, thereby bypassing the TCA cycle. While it is possible that this interpretation is incorrect, it would be helpful to see it addressed in the manuscript.

We agree that this is a plausible hypothesis, we have added a paragraph at the end of this section that discusses the implications of overflow metabolism as an alternative hypothesis.