# Controlled Measurement and Comparative Analysis of Multiple Cellular Components in *E. coli* under Long-Term Starvation

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## Abstract

Genome wide models are a useful tool in synthetic engineering of biology. Most genome-wide models however focus on individual subsystems, or networks, of the cell, such as in flux balance models of metabolism. Recent effort has been focused on how to best integrate models of separate subsystems (e.g. transcription, metabolism, etc.). Thus, there is a need for consistent and comparable measurements of these different cellular components. Though many large data sets exist for specific types of cellular components (e.g. RNA expression, protein abundances, and metabolites) there is a lack of data that span multiple different sub-systems under the same controlled conditions. Here we present genome-wide measurements on RNA and proteins as well as lipids and metabolic fluxes from E. coli grown under identically controlled conditions. These data serve as a resource for building integrative models of cellular processes. Specifically, we present data from a long-term glucose starvation time course. These data comprise the first completed results from a series of planned experiments considering different environmental conditions. Furthermore we characterize and discuss the general trends seen in this time course comparing and contrasting our data sets on different sub-processes.

# Introduction

Many global changes in cellular physiology occur during the growth of a typical laboratory culture of a microorganism, such as *E. coli,* as it reaches an exponential rate of cell division after an initial lag phase and then eventually ceases dividing when nutrients are exhausted (Neidhardt and Curtiss, 1996). However, how these changes affect specific cellular components and processes is not fully known. Existing works, even if done at the genome scale, tend to have limited completeness, in at least two ways. First, most studies consist of the piece-wise collection of only one type of genome-scale data. Second, technological limitations often prevent the detection of some subset of molecules in a category of interest. For example, small bacterial RNA species with key roles in regulation are lost when using typical purification methods (Stead et al., 2012). Furthermore, DNA microarray-based methods for profiling gene expression can only detect specific RNA sequences depending on the design of their probes, whereas RNA-seq transcriptomic methods theoretically recover all RNA species in a sample (Wang et al., 2009). Similarly, in proteomics, 2-D gel electrophoresis approaches typically detect many fewer proteins than newer mass spectrometry based shotgun methods do (Soares et al., 2013; Wiśniewski and Rakus, 2014).

Moreover, while the short-term changes in cellular physiology that occur in a laboratory culture of *E. coli* have been the subject of intensive study, considerably less is known about the changes in cellular composition that occur during the long-term survival of *E. coli* and other non-spore-forming microbes under starvation, despite the likely prevalence of this condition in nature (Morita, 1990). Most studies of this metabolic state have concentrated on using rich media in which growth ceases and there is an ecological catastrophe accompanied by the death of most cells and the emergence of mutants that are able to continue dividing (Finkel and Kolter, 1999). Thus, these are often studies of evolution to new nutrient sources rather than purely of cellular physiology in stressed, starving, but wild-type cells. The specific physiologic changes that occur in *E. coli* under long-term starvation are thus not well understood.

Here we performed a time course experiment of *E. coli* B REL606 growth and starvation up to two weeks. We used a chemically defined glucose-limited medium in which cells enter a starvation state but do not die. We collected genome-wide RNA and protein levels, lipid modifications, and metabolic-flux data at multiple time points, all under identical, controlled experimental conditions. The resultant data set serves as a rich resource for computational models that span and integrate cellular sub-systems and for cataloguing and correlating the responses of specific genes and/or molecules across cellular subsystems during growth and long-term starvation. We find that post-translational regulation can play a role in buffering proteins with consequences to regulation of *E. coli* physiology, that the mRNA pool is drastically reduced during starvation, possibly to limit new protein synthesis overall, and that *E. coli* are able to persist in stationary phase for over a week. Additionally, we propose a general approach for unbiased classification of expression time courses that could aid in analyzing future gene expression and proteomic time courses.

# Results

## Controlled measurements of multiple cellular components

We grew multiple culturesof *E. coli* REL606, from the same stock, under identical growth conditions of long-term glucose starvation, in the same media. The cultures were subsequently distributed to different labs to measure RNA, protein, lipids, and metabolic flux ratios (Figure 1A). Freezer stocks of the REL606strain where revived for 24 hrs, diluted and preconditioned for another 24 hrs, and diluted again at the start of the experimental time course (Figure 1B). In a pilot experiment a growth curve was taken to determine informative time points for analysis (Figure 1C). Time points spanning 3 hrs to 2 weeks where collected and equitably distributed to measure RNA via RNAseq, proteins via LC/MS, lipids via MALDI-TOF MS and ESI MS, and Metabolic Fluxes via U-13C labeled glucose and GC-MS (Figure 1D). <We need to comment on the viability of cells at late times here, or at least somewhere in this sub-section. Cells don’t die much in our setup.>

We first assessed reproducibility of our data, and found that it was highly reproducible. Biological repeats of protein and RNA measurements correlated highly with each other. We saw Pearson correlations of 0.95 and 0.92 between biological repeats of raw proteomics counts and correlations of 0.93 for raw RNAseq counts between biological repeats (Figure 1-supplemental 2). We next compared coverage relative to previous works, and found that we obtained more in depth coverage compared to other similar studies that measure both protein and RNA (Table 1). Yoon et al. used 2D gels and microarrays to measure 60 significantly changing proteins and 4144 mRNAs in the *E. coli* REL606 strain, the same strain used in this study (Yoon et al., 2012). By comparison, we observe over 2600 proteins with ~1200 that change significantly through the duration of our time course along with 4116 mRNAs, 89 sRNAs, 85 tRNAs (at early exponential phase). Even though the total number of proteins in Yoon et al. observed at early exponential phase is not reported, it is likely an order of magnitude less than our observations, if it follows the same pattern as the proteins undergoing fold change. Taniguch et al. measured protein and mRNA content of single cells using YFP fusions and FISH, resulting in the measurement of 1,018 proteins and 137 transcripts in an *E. coli* K12 variant (Taniguchi et al., 2010). Lewis et al. also measured ~1,000 proteins and RNA expression of 4428 genes. Although these data sets where published separately, they where performed in the same lab and under similar conditions and thus are also comparable to a degree (Lewis et al., 2010, 2009). Our proteomics measurements are far more complete than other comparison studies, providing more than 1,000 additional protein observations than the previous top comparative study, as many mRNAs as other studies, and additional data on tRNAs and sRNAs.

Our experiments also provide coverage comparable to or better than other experiments that focus on proteomics or RNA measurements alone. Using SILAC, Soares et al. observed 2,053 proteins in at least 1 of 2 biological repeats, at a false discovery rate (FDR) of <1% (Soares et al., 2013). We measured 2,658 proteins in at least 1 of 3 biological repeats with around 2,200 protein IDs per sample using the same FDR cutoff. A more recent study, using the FASP method, also observed around 2,200 proteins per sample, comparable to our recovery (Wiśniewski and Rakus, 2014). Additionally, using RNAseq, we recovered as many mRNAs as microarray approaches do, with the added benefit of measuring 89 sRNAs and 85 tRNAs at the same time (Raghavan et al., 2011). As a point of reference, previous RNAseq experiments on the *E. coli* K-12 strain found 133 sRNAs and 4161 genes. Thus our recovery of both proteins and RNA represent the state of the art of the field, far outperforming recent comparative studies. As an added benefit of our study we also simultaneously measured lipidA, phospholid, and flux ratios in central metabolism, covering a wider range of cellular components than previous comparison studies.

## General trends in RNA and protein abundance over time

We next investigated changes RNA and protein abundances over time. Gene expression is often differentially regulated in response to changes in the environment. Due to translational and post-translational regulation we expected differences in the response of RNA transcripts and proteins in response to glucose starvation. To visualize these differences we compared and contrasted the general trends in the response of RNA and proteins by way of *K*-means clustering. To simplify the analysis we focus on only those RNAs and proteins that are changing significantly (as measured by false discovery rate and fold change cutoff, respectively) throughout the time course, yielding ~1900 significantly changing transcripts/proteins. *K*-means clustering requires an arbitrary choice for the number of clusters, chosen such that the profiles are well separated into groups with unique and distinct behaviors. (In subsection “RNA and Protein time course classification”, we will propose an alternative classification approach that does not depend on such an arbitrary choice.) We varied the number of clusters for both RNA and protein profiles to find a satisfactory grouping of the data, and we found the best clustering performance to be around 15 clusters for the RNA profiles and 25 clusters for the protein profiles. Thus, the RNAs respond in a much more uniform manner than the proteins do. This is illustrated by the heatmap of the cluster centroids of RNA and protein (Figure 2A and B, respectively). A vast majority of the RNAs are down-regulated while the protein response is much less uniform, likely due to differences in post-translational modification such as degradation. Additionally, the RNA profiles show a clear separation between early and late time points with a transition period around 6-8hrs. The transcription profiles remain relatively constant, with only minor changes in expression, after this transitional period of entry to stationary phase. At two weeks some of the transcripts begin changing again perhaps signaling a further shift in the cells state.

We would like to emphasize that the above clustering of the RNA and protein abundances are independent and thus we cannot directly compare individual clusters between Figures 2A and B. The next section addresses the correlation between absolute and relative changes in abundance of individual proteins and their transcripts.

## Correlations between individual mRNAs and proteins

It is well known that absolute levels of proteins do not correlate strongly with their corresponding transcripts. In addition to a weak absolute correlation between mRNA and protein at a given time point we also expected, due to potentially long protein degradation rates in stationary phase, a weak relative correlation within a single time course between a protein and its transcript. To put proteins and RNA within a given sample on comparable scales, we normalized protein counts using the apex method (Lu et al., 2007) for absolute quantification and the mRNA to the length of each transcript. Both protein and mRNA levels were then averaged across all three biological replicates. Additionally, all proteins and mRNAs were scaled by the average of all proteins and mRNA.

To relate the relative levels of a protein to its transcript we had to account for the underlying dynamics of the time courses. We considered two limiting cases: At one extreme we assumed each protein has a degradation rate slower than the time scale of the experiment. At the other extreme we assumed each protein is degraded on a time scale that is fast compared to the time scale of the experiment. In the first limiting case proteins integrate their transcript levels over time. In the second limiting case (relative) protein levels track with their (relative) transcript level. If a protein is not integrally or proportionally related to its transcript it could be due to an intermediate protein degradation rate, saturation effects, or some other post-translational regulation or nonlinearity.

The strongest absolute correlation, across the time course, between mRNA and protein was at three hours (Figure 2F, Spearman ρ=0.59, *P*=10-224). This number is in line with measurements of correlations between RNA and protein for other measured prokaryotes and eukaryotes, which have Spearman correlations around 0.5 between proteins and their transcripts (Abreu et al., 2009; Gygi et al., 1999; Vogel et al., 2010; Vogel and Marcotte, 2012; Washburn et al., 2003). Plotted in Figure 2C and D are histograms of the Spearman correlation coefficients (ρ) calculated for protein vs. the integral of its transcript and protein vs. its transcript, respectively. Approximately 15% of the proteins correlated highly (ρ>0.75) with the integral of their transcripts whereas approximately 20% correlated highly with proportional levels of RNA. There is no overlap between the two sets as can be seen by the strong anti-correlation in the 2D histogram in Figure 2E of protein vs the integral and proportional levels of mRNA. Genes that are proportionally regulated are enriched for, among other things, locomotion and cell division. Genes that are integrally regulated are enriched for glycerol, aditol, and polyol metabolism. For a full list of gene ontology enriched terms see supplemental figure3-supplemental table 1. For a full list of proteins that are either proportionally or integrally related to their transcripts see figure3-supplemental table 2.

## Population shifts of RNA pools in long-term glucose starvation

As cells enter stationary phase overall demand for overall new protein synthesis is significantly decreased, demand for certain stress response proteins increases, and resources become limiting. New protein synthesis can be globally limited in at least three ways: reducing the overall available rRNA, tRNA, or mRNA. To understand how these different RNA pools change relative to each other, we calculated the relative amount of mRNA, tRNA, sRNA, and rRNA present in both ribosome depleted and non-ribosome depleted samples (Figure 3A and B). In the non-ribosomal depleted case the fraction of rRNA changes very little throughout the course of the experiment while the tRNA fraction increases and the mRNA fraction decreases. In the ribosome depleted samples, the tRNA fraction also increases as the mRNA fraction decreases confirming that this effect is not due to sensitivity issues resulting from rRNA dominating the RNA pool in the non-ribosome depleted sample.

## Correlation of expression between genes in the same operon

Genes within an operon are co-transcribed as a single RNA and thus are likely to be under the same transcriptional control. Differences in translational efficiency between genes, localization, feedback, or other post-translational modifications often lead to differences in protein expression of genes in the same operon (Lim et al., 2011a; Mattheakis and Nomura, 1988; Wek et al., 1987; Yamada and Saier Jr, 1988). It has been shown that there is a correlation between the translational efficiency of a gene and its genetic distance from the end of the operon (Lim et al., 2011a). We expect to see very high correlation between RNAs within an operon as they are under the same transcriptional control; however, we expect there to be less correlation between proteins within an operon as proteins within an operon are not guaranteed to be under the same translational/post-translational regulation.

As a measure of correlation of gene expression within an operon we took the average of the pairwise Pearson correlation coefficient for all possible pairs of transcripts and proteins within an operon. Approximately eighty percent of transcripts had a mean pairwise correlation coefficient greater than 0.8 within an operon (Figure 4A). On the other hand less than fourteen percent of proteins had a mean pairwise correlation coefficient greater than 0.8 within an operon (Figure 4B). Genes closer together within an operon are more likely to have correlated protein profiles (see Figure 3C), which we take as evidence that distance between genes is a strong indicator of translational regulation.

We also show a few examples of highly correlated transcript and protein proteins for individual operons (Figure 4E and C/D respectively). Even though the proteins in these plots have, on average, a high pairwise correlation there are still some differences in the expression profiles, unlike the transcripts that have nearly identical expression profiles for a given operon.

## RNA and Protein time course classification

Typical analysis of RNA expression data often involves performing a hierarchical clustering of profiles followed by a term enrichment of subsets of genes found in the emerging patterns. In this approach the patterning that comes from hierarchical clustering can be arbitrary, dependent on what level of the hierarchy one chooses to focus on. Thus, we sought to sort the time courses into general behaviors in an unbiased manner. To accomplish this we fit each individual mRNA and protein to a piecewise continuous curve (Figure 5A). This curve is defined by 4 free time parameters and 3 free amplitude parameters. To fit the curve we used a population based differential evolution (DE) algorithm. Furthermore the fitness function used in minimization is scaled to the experimental error (see methods). Thus, our algorithm provides confidence intervals for our fit based upon the variability in biological replicates. To demonstrate the effectiveness of our fitting strategy we randomly selected five mRNA profiles and their respective fits (Figure 5B-F). Green circles show the average of three biological replicates with their standard deviations (green bars) and the blue line and bar show the average and standard deviation of the population of fits respectively. Both the data and fit have been normalized to the average of the time course.

As can be seen in Figure 5B-E, for mRNAs we generally get good agreement between the data and model. Thus, the fits give us reasonable estimates of the distribution of time scales involved in the response. Figure 5F shows the distribution of *t*1, the time to first inflection. Most of the mRNAs respond between 3-8 hrs, with a strong peak at around 6 hrs (when cells begin entry to stationary phase). To better understand the regulation of cellular processes (and mRNAs) in our dataset, we sorted the mRNA profiles, based upon the estimated parameters of our fit, into five general categories: up-regulated, down-regulated, temporarily up-regulated, temporarily down-regulated, or unknown. The confidence intervals for our fits allowed sorting individual mRNAs into these five categories with high confidence. The mRNAs in the categories “down-regulated” and “up-regulated” showed significant enrichment for GO terms. The average of the mRNAs in each of these terms can be seen in Figure 6A, B. Terms enriched in the set of down-regulated transcripts are involved in translation, carboxylic acid biosynthetic process, and nitrogen compound biosynthetic process. These processes are likely down-regulated for energy conservation purposes in the face of limiting resources. Terms enriched in the set of up-regulated transcripts are involved in carbohydrate catabolic process and alditol metabolic process. These two terms are involved with the breaking down and processing of carbohydrates.

To characterize the protein response we followed the same general strategy of fitting, classification, and GO enrichment as we did for the RNA profiles. The distribution of the time to first inflection for the proteins is a little broader than the mRNAs. However, they are still mostly in the range between 3-8 hrs with very few proteins that have not responded by the time the cells enter stationary phase. There are many proteins that are turned on for the duration of the time course, compared to the mRNAs where very few remain on for the entire duration of the experiment. Figure 6D shows the average of the proteins in a given GO term that are enriched in the set of proteins that are being up-regulated. As in the case of down-regulated RNAs these proteins are likely down-regulated to conserve energy, and they include proteins involved in translation and locomotion. Up-regulated proteins are, like the up-regulated transcripts, involved in carbohydrate catabolism but also include terms involved in stress response and metabolism of glycerol. The average of proteins in GO terms being down-regulated have a much wider distribution of decay times compared to the RNAs being down-regulated likely due to differing protein degradation rates (and/or thermodynamic stability) (Figure 6C).

As a complementary approach we also averaged all proteins in a given Kegg pathway regardless of their behavior. Many pathways showed little to no differential regulation, on average, in their protein levels. Pathways that did change cohesively are plotted in Figure 6E and F depending on if they were down or up-regulated, respectively. As in the previous term enrichment analysis, we see motility down-regulated as well as other energy consuming processes involved in metabolism and biosynthesis. Interestingly biosynthesis of siderophores was up-regulated, likely due to do increased demands for, or reduced supply of, iron.

## Flux ratio analysis.

We used flux ratio analysis to measure the relative metabolic fluxes passing through different branches of the central metabolism. To measure flux we relied on the amino acid labeling pattern. As there is little ab-initio protein synthesis after the cells stop growing (after ~8hrs) the computed flux ratio after eight hours represent a cumulative flux ratio. Thus after this time we expect that most of the flux ratios to remain relatively constant and this is certainly the case. The only significant changes in the flux ratio that occur during the time course are in P5P from G3P and S7P and P5P from G6P lower branch (Figure 7G). P5P from the G6P lower branch decreases from 1 to 0.5 into the second week. P5P from G6P and S7P is one minus the flux from P5P from the G6P lower branch. All other measured flux ratios showed little change through the course of the experiment (Figure 7A-I).<representative more of low protein turnover than anything else?> We also compared the flux ratios with the corresponding ratio of enzymes responsible for catalyzing those reactions both at the level of RNA and protein. Generally speaking, the protein ratios changed little, consistent with the low change in flux ratio <Note: I’m not sure if this is true, Viswanadan will have to speak to this>. The flux ratio that did change (P5P from G6P lower branch), however, showed a strong correlation with its corresponding protein ratio (ρ=0.85, *P*=…) and a weak (but significant?) correlation between its corresponding mRNA ratio (ρ=0.5, *P*=…).

## Lipid profiles

Using negative-ion MALDI-TOF and ESI mass spectrometry (MS), we analyzed lipid A and phospholipid profiles of cells at each time point, respectively. Beginning before one week, we observed an appearance of an MS peak associated with the acylation of lipid A with a C16 chain (Fig. 5C). In the phospholipid analysis, a notable increase began around 8hr in the cyclopropanation of one unsaturated double bond within molecules of the major phospholipids, phosphatidylethanolamine (PE) and phosphatidylglycerol (PG). This was identified by the gradual relative increase of peaks at ~702.5 m/z and XXX m/z, respectively (representative data for PE is shown in Fig. 8D). Both the modifications to lipid A and phospholipids continued to increase up to the 2 week period. In fact, the 702.5 m/z peak corresponding to cyclopropanation of phospholipid was barely detectable before six hours but became the predominant peak by the end of the time course.

The enzymes relevant to the above lipid A and phospholipid modifications are PagP and cycloproponated fatty acid synthase (CFA), respectively (Bishop et al., 2000; Grogan and Cronan, 1997). PagP is known to be constitutively transcribed at low levels and remain latent in the outer membrane until enzyme activation (Jia et al., 2004). It is also up-regulated by the transcriptional regulator, PhoP, under various stressful conditions encountered by a cell (Needham and Trent, 2013). However, during our time course, transcript levels of PagP and PhoP did not change significantly. Furthermore, neither PagP or PhoP was observed at the protein level. In the case of PagP, this could be due to the difficulty in detecting outer membrane beta-barrel proteins. With respect to phospholipid modification, CFA synthase protein levels increase between 3-6 hrs before decreasing again. This correlates with prior data that CFA synthase is important for transition to stationary phase of growth (Grogan and Cronan, 1997). CFA synthase RNA levels increase again around 1 week, which is consistent with the activity observed in phospholipid modification, although it is not known why we did not observe a corresponding increase in protein levels at this point (Fig. 8B).

# Discussion

By making controlled measurements of both RNA and protein under long-term glucose starvation, we made several novel observations. Strikingly, *E. coli* can survive for over a week when starved for glucose, with little change in cell viability and optical density (at 600nM). Additionally, all genes start to change in expression by 10hrs and mRNA expression clusters in time into two regimes before and after 10hrs (cells enter stationary phase at around 8hrs). We also found that proteins involved in cell division and energy intensive processes such as cell locomotion where proportional to their transcripts allowing for a rapid down-regulation of these processes. On the other hand, proteins integrally related to their transcripts where enriched for metabolic processes allowing cells to rapidly make use of any nutrients that may become available again without the continued cost of making these proteins. In addition to measuring and characterizing RNA and protein changes under starvation, we also proposed a novel fitting strategy that allowed us to classify expression profiles into different categories in an unbiased manner. The enriched terms in the resulting classification where reasonably aligned with what is known, or at least consistent with, cells coping with starvation. Importantly, this classification was accomplished without any ad-hoc determination of cluster number that could introduce bias in interpreting the data.

We have found that, as cells enter stationary phase, the total pool of mRNA is depleted compared to all other RNAs and many individual transcripts are down-regulated, possibly as part of a broader strategy to reduce the production of new protein. There are at least two additional ways of reducing overall protein production, by limiting the available ribosomes or by limiting the pool of available tRNA. It is known that during nutrient limitation a stringent response is activated, through ppGpp, and synthesis of new rRNA is down regulated, presumably to help reduce new protein production (Magnusson et al., 2005). In our data, the fraction of rRNA changes little while the tRNA fraction increases as the time course goes on. Thus new protein synthesis could be limited more by the reduced mRNA pool than by reduced translational efficiency.

It is widely appreciated that the half-life of many proteins in *E. coli* is rate limited by the division rate of the bacteria. Thus the degradation rate of most proteins in *E. coli* must be slower than the division rate in exponentially growing cells. As a consequence, when cells cease to divide, such as in the case of glucose starvation, not all proteins can respond immediately to possible changes in transcript levels. In effect, some proteins may be buffered to relatively fast changes in nutrient availability. At the same time certain proteins may need to be rapidly regulated to ensure survival upon starvation. We found that a small subset of the proteome, ~20% of proteins, fell into the rapidly regulated category and were proportional to their transcripts. Another small subset, ~15% of proteins, tended to integrate the response of their transcripts over the time scale of our experiment. For example, several of the flagella proteins are proportional to their transcripts, whereas proteins involved in metabolism and energy production integrate their transcript levels. Turning off proteins involved in cell division and the flagellum machinery, both energy intensive processes, needs to happen relatively fast. By contrast, the proteins that are relatively stable are enriched for energy production terms. Thus, these proteins presumably persist so that once nutrients become available again the cell can immediately take advantage of them.

We found that several genes (both among the protein and the RNA profiles) that where significantly up- or down-regulated during starvation where uncharacterized or had no annotation. A subset of these uncharacterized proteins have computationally predicted functions (Hu et al., 2009). Some of these predictions are consistent with our findings for annotated genes. For instance, several uncharacterized proteins that are up-regulated are predicted to be involved in stress response and cell-wall bio-genesis. Other predictions seem to be inconsistent with our observations for annotated genes. For example, some uncharacterized proteins that are up-regulated are predicted to be involved in translation, even though translation was heavily enriched in down-regulated genes. Lists of proteins, and transcripts, that are significantly regulated in our time course are provided in the supplemental materials (Tables SX) and include behavior (e.g. up/down-regulated), curated annotations, and functional predictions (where available).

Though transcripts within an operon are highly correlated, in many cases their protein profiles are only weakly correlated. This could be due to different translation efficiencies between proteins (Lim et al., 2011) as well as differing degradation rates. In support of the former we see a tendency for proteins separated by a larger genetic distance to be less correlated than those closer genetically. However it is likely that different protein degradation rates also play a role in the low correlation between proteins within an operon. Indeed many genes that are close genetically also show poor correlation in their profiles (Figure 3C)

In addition to the expected disparities between RNA and protein levels, we also observed surprising changes in enzyme activity that did not correspond to the respective RNAseq and proteomics analysis. For example, we saw striking levels of lipid modification late during the time course. These modifications are easily explained by their association with adaptation to stressful environments such as depleted nutrients and cations as well as increase acid resistance during starvation (Grogan and Cronan, 1997; Needham and Trent, 2013). However, the stark differences in RNA, protein and activity trends of the enzymes responsible for the lipid modifications, PagP and CFA synthase highlights the fact that activation does not necessarily follow abundance measurements.

A goal of systems biology has been to understand how phenotype originates from genotype. The phenotype of a cell is determined by complex regulation of cell signaling, gene regulation, metabolism, and lipid biochemistry. Understanding the connection between phenotype and genotype is crucial to understanding disease and for synthetic engineering of biology. Computational models are particularly well suited to studying this problem as they can synthesize and organize diverse and complex data in a predictive framework. Even though computational models of individual component subsystems, such as flux models of metabolism (Duarte et al., 2004; Feist et al., 2007; Feist and Palsson, 2008), have enjoyed a long history of success, they remain limited in their application. Much effort is currently being spent on understanding how to best integrate multiple subsystems. For example, there are many proposed approaches to combining gene expression with metabolic flux networks (Åkesson et al., 2004; Colijn et al., 2009; Collins et al., 2012; Fang et al., 2012; Lee et al., 2012; Mahadevan et al., 2002; O’Brien et al., 2013; Van Berlo et al., 2011; Vogel and Marcotte, 2012) while other studies are focusing on integrative, whole-cell, models (Carrera et al., 2014; Karr et al., 2012). Given the growing interest in integrative modeling approaches, there is a pressing need for studies that collect high quality genome-wide data across multiple cellular subsystems from the same biological samples. Our data set is a rich resource for comparing and contrasting the response of multiple cellular subsystems.

Despite the completeness and quality of our data set, however, there are a few key limitations concerning our approach. Our analysis via RNAseq and shotgun MS allows for high confidence in comparing the relative levels of a particular transcript or protein over time. However, due to potential differences in detection efficiency between individual RNAs or peptides, care should be taken when comparing absolute abundances. In our analysis we used the APEX method to account for differences in detection efficiency. We normalized RNA by the length of a transcript as an estimate of RNA detection efficiency, for a particular experiment. This resulted in a correlation coefficient of ~0.6 between proteins and their transcripts, a finding on the high end for such correlation measurements. Thus, even straightforward means of correcting our experimental bias can potentially lead to reasonable comparisons of levels between individual RNAs or proteins.

Though the stepwise linear function we used used for modeling works for a majority of our expression profiles, in some cases it over-fits the data and in other cases the function is unable to capture the underlying behavior. An example of a profile that may be under-constrained is a gene that is up- or down-regulated without further changes to expression. In this case the free time parameters *t*2 through *t*4, along with amplitude parameter *A*2, may be under-constrained. Even in this case, however, the parameters *t*1, *t*2+*t*3+*t*4, *A*1, and *A*3 are still well constrained, providing enough information to reliably sort the profiles based upon behavior. More complicated behavior, such as multiple peaks separated in time, cannot be captured by our function. The presence of these more complicated behaviors was rare enough as to not warrant special consideration.

# Methods

## Growth procedure

*E. coli* B REL606 was inoculated from freezer stock in 50mL DM500 and incubated at 37C overnight. 500 uL of the overnight culture was diluted in 50mL of DM500 at 37C and grown for 24hrs. On the day of the experiment, 500UL of the 24hr culture was added to 10 flasks containing 50mL DM500 each, grown at 37C. At each time point 1ml was removed from the flasks, washed with 0.7% NACL, spun down, the supernatant was removed, and the remaining cell pellet was flash frozen using liquid nitrogen and stored at –80C.

## RNAseq

Total RNA was isolated from cell pellets using RNAsnap method . After extraction, RNA was ethanol precipitated and resuspended in 100ul ultra pure water. The RNA was then cleaned and DNase treated using Zymo Clean and Concentrator 25 (Zymo Research). RNA was then eluted in 50ul ultra pure water. Total RNA concentration was determined using Qubit 2.0 Fluorometer (Life Technologies). Ribosomal RNA was depleted using Gram-negative bacteria RiboZero rRNA removal kit (Epicentre). After depletion the RNA was ethanol precipitated and resuspended in 20ul ultra pure water. RNA concentration was determined using Qubit 2.0 Fluorometer (Life Technologies). RNA was then fragmented to approximately 250 bp using NEBNext Magnesium RNA Fragmentation Module (New England Biolabs). After fragmentation the RNA was ethanol precipitated and resuspended in 20ul ultra pure water. After fragmentation, the RNA was Kinase treated using T4 PNK (New England Biolabs). The RNA was ethanol precipitated and resuspended in 20ul ultra pure water. Library preparation was done using NEBNext Small RNA Library Prep Set for Illumina, Multiplex Compatible (New England Biolabs). Each sample was multiplexed using NEBNext Multiplex Oligos for Illumina (New England Biolabs). After library preparation samples were ethanol precipitated and then run on a 4% agarose gel. All DNA greater than 100 bp was excised and cleaned using Zymoclean Gel DNA Recovery kit (Zymo Research). Final library concentration was determined using Qubit 2.0 Fluorometer (Life Technologies). Libraries were sequenced using an Illumina HiSeq 2500 at the Genomic Sequencing and Analysis Facility (GSAF) at the University of Texas at Austin.

For RNA-seq analysis, we used our own custom analysis pipeline using the REL606 Escherichia coli B strain genome as the reference sequence (Jeong et al., 2009). Prior to mapping, all of the single-end reads have had the adapters trimmed using FLEXBAR 2.31 (Dodt et al., 2012). Mapping was carried out using bowtie2 2.1.0 with the –k 1 option to achieve one unique mapping location per read (Langmead and Salzberg, 2012). Raw number of reads mapping per gene were counted using htseq-count 0.6.0 (Anders et al., 2014). The exact pipeline is available at <https://github.com/clauswilke/Ecoli_RNAseq>.

## Proteomics

Frozen pellets where resuspended in 300 uL of buffer (50mM Tris-HCL pH 8.1,100mM KCL, 5mM MgCl2). 50UL of sample was removed for preparation,treated with 50uL trifluoroethanol (TFE), and placed on ice for 15min. DTT was then added to a final concentration of 5mM and incubated at 55C for 45 min. Samples where alkylated by addition of Iodoacetamide (IAM) to a final concentration of 15mM. Trypsin digest was performed by addition of 800uL of 50mM Tris pH 8.0, 2mM CaCl2 followed by 2ug of trypsin. Digestion took place at 37C for 4-5 hrs and was stopped by 10uL of formic acid (1% vol./vol). Samples where then Ultrafiltrated to remove insoluble and undigested material using Amicon Ultra MWCO 10kD spin-caps and finally concentrated and purified by C18 filtration.

Liquid chormotography and mass spectrometry (LC/MS) was carried out on a LTQ-Orbitrap (Thermo Fisher) as has been described previously <ref>. <particular settings need to be filled in>

We searched the raw MS-spectra using a Sequest search against a database for *E. Coli* REL606 that contained reverse, decoy, sequences to help control for false positives.

## Flux analysis

Flux ratios were obtained using the methods of (Zamboni et al., 2009). Cell pellets were resuspended in 200 mL of 6 N HCl, hydrolyzed at 105°C overnight, and dried at 95°C for up to 24 hours. To the hydrolyzed cell material we added 40 mL of dimethylformamide (DMF) and gently mixed until a “light straw” color was obtained. The DMF resuspension was transferred to a GC-MS vial with plastic insert and 40 mL of N-tert-butyldimethylsilyl-N-methyltrifluoroacetamide with 1% tert-butyldimethyl-chlorosilane; vials were capped and baked at 85°C for 2 hours, and samples were analyzed within 2 days of derivitization.

Analysis of derivitized samples was performed on a Shimadzu QP2010 Plus GC-MS (Columbia, MD) with autosampler. The GC-MS protocol included: 1 mL of sample injected with 1:10 split mode at 230°C; an oven gradient of 160°C for 1 min, ramp to 310°C at 20°C/min, and hold at 310°C for 0.5 min; and flow rate was 1 mL/min in helium. A total of five runs were performed for each sample: a blank injection of DMF to waste, a blank injection of DMF to the column, and three technical replicates of each vial.

Details of flux inference?

## Lipid analysis

Lipid A and phospholipids were isolated from bacterial pellets containing 3-9 x 109 cells. Pellets were resuspended in 5ml 1:2:08 CHCl3: MeOH: H2O for 20 minutes and spun at 10,000 xg for 10 minutes. Pellets containing lipid A were further purified by the Bligh/Dyer method as previously described (Hankins et al., 2013). Phospholipids in the supernatant were further purified by extractions as previously described (Giles et al., 2011). Mass analysis of purified lipid A fractions was performed using a MALDI-TOF/TOF (ABI 4700 Proteomics Analyzer) mass spectrometer in the negative ion linear mode as previously described (Hankins et al., 2013). Phospholipid analysis was performed by liquid chromatography/ESI-mass spectrometry at Duke University as previously described (ref?).

## Expression profile data analysis

This section outlines our process for analyzing raw counts from the proteomics and RNAseq experiments. Initially, proteins with low counts (<10) over the entire duration of the time course where filtered out. Each time point was then normalized to the read depth (e.g. the sum of all counts for that particular time point). Only proteins with a fold change of >=1.5 where considered for further analysis. Protein profiles where then normalized to the maximum value for a given protein time course. To estimate the absolute protein abundance we made use of the APEX normalization method (Vogel and Marcotte, 2008).

Raw RNA counts where normalized using the DeSeq method (Anders and Huber, 2010). RNAs with a *p*-value less than 0.05, at some point during the time course, where considered to have changed significantly and kept for further analysis. To compare RNA abundances within a single time point, raw RNA counts where normalized to their read depth (sum of all counts for that time point). Finally, normalized RNA and protein profiles, both relative and absolute, where averaged across all three biological replicates.

Clustering of protein profiles was performed using the python library scipy (Jones et al., 2001). We used the *k*-means clustering algorithm with the number of protein clusters set to 25 and RNA clusters to 15. To compare relative protein profiles with the integral of their relative transcript levels we integrated each of the transcript profiles, from the initial time to each additional time point, using the trapezoidal method implemented by the python library numpy (Walt et al., 2011).

We used a piecewise continuous curve to fit both RNA and protein profiles. This curve was defined by seven free parameters, 4 free time parameters, and 3 free amplitude parameters. To fit the profiles we used a custom implementation of a differential evolution (DE) algorithm(Price et al., 2005). Briefly, the DE algorithm generates an ensemble of random parameter guesses within a predefined range, at each iteration vectors of individual parameter sets (sometimes called agents) are mixed together at a predefined crossover rate,only those crossover events that yield a smaller error (defined by a predefined cost function) are kept, and the process is iterated until a convergence criteria is met. In our fits we used an ensemble of 15 agents with a crossover frequency of 0.75 and an mixing strength of 0.6. The crossover frequency determines the probability that an agent will be changed at any given iteration and the mixing strength determines how large a change an agent undergoes if it is chosen to be altered. The crossover frequency and mixing strength where picked based upon an empirical study of the dependence of convergence efficiency on these parameters (Pedersen, Magnus Erik Hvass, 2010) for some standard optimization problems. The cost function is given by



where *di*(*tj*), *σi*(*tj*), and *si*(*tj*) are the average of all experimental repeats of protein (or mRNA) *i* at time *tj*, the standard deviation of the experiments of the protein (or mRNA) *i* at time *tj*, and the average of the ensemble simulations *i* at time *tj*, respectively. Scaling by the standard deviation places a relatively lower weight on data points with relatively larger errors for a given protein or mRNA.

Some of the profiles may be slightly over fit by our curve (e.g. profiles that are upregulated or down-regulated once during the time course without further modulation of expression). Thus care needs to be exercised in the interpretation of some of the parameters. However, we found *t*1 to reliably give the time to first inflection, that the sum of *t*2, *t*3,and*t*4 was a decent proxy to how long it took an RNA/protein to reach a steady state after entering stationary phase, and that we could reliable sort the behavior into four categories based upon the amplitude parameters. The four categories we used where that of up-regulated, down-regulated, temporarily up-regulated or temporarily down-regulated. The sorting into categories was aided by our estimate of the distribution of parameters that allow for a good fit within in the population of fits. A fit was considered good if it was on average (across the time course) one standard deviation, or less, away from the experimental average.

We used the DAVID database (david.abcc.ncifcrf.gov) to perform Gene Ontology term enrichment on each subset of sorted genes: up-regulated, down-regulated, temporarily up-regulated or temporarily down-regulated. Specifically we made use of DAVID's API, instead of the web interface, to generate the GO-enrichment through a python script. GO terms where clustered based upon genes in a given term to reduce redundancy in the returned results.

As a complementary approach we also enriched, for Kegg pathway terms, the entire set of significantly changing proteins (without presorting) using, again, the DAVID database API. The proteins within each returned Kegg pathway where then averaged to see if there was any consistent response across the entire pathway. Those Kegg terms that gave in-cohesive responses across proteins in that pathway returned a relatively flat average and where filtered out.

All of the scripts used to perform the above analysis can be downloaded at github.com/marcotte/AG3C <I should merge Daryia's code with mine>.

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# Figure Captions

**Figure 1.** **Overview of experimental work flow.**

(A) Measurements of RNA, protein, lipids, and metabolic flux are taken under uniform growth and environmental conditions. (B) Long-term glucose starvation experiment. *E. coli* B REL606 strain was taken from freezer stock and revived in 10ml DM500 media with minimal glucose for 24 hrs (day -2). The next day this culture was diluted 1:10 in 500mL DM500 and grown for 24 hrs (day -1). On the day of the experiment (day 0) the previously conditioned culture was diluted again into several individual cultures. (C) The OD @ 600 was measured to assess growth and optimal collection of time points. Nine time points where selected for this experiment spanning 3hr-2wks. (D) For each sample 1ml was removed from the culture for each experiment to be done, spun down, flash frozen, and distributed to the experimental labs in our consortium to measure RNA via RNA seq, protein via LC/MS, lipids via Maldi-TOF MS and ESI MS, and metabolic flux via GC-MS.

**Figure 2. *K*-means clustering of RNA and protein profiles in long-term glucose starvation**

RNA and Protein profiles, normalized to each molecules maximum value, where clustered by geometric distance using *K*-means clustering with 15 and 25 clusters respectively. The cluster centroids where then plotted as heat maps with darker blue representing higher RNA, or protein, levels. (A) RNA levels are largely shutoff upon glucose starvation with some RNA being temporarily up-regulated during the transition between exponential and stationary growth. (B) Protein levels show a much wider range of behaviors with some being up or down-regulated for the duration of the experiment as well as for a short period of time during the transition from exponential to stationary growth. (C) Histogram of the correlation coefficients between individual protein levels and the time integral of RNA. This is the limit of slow protein degradation where protein levels are proportional to the cumulative sum of their respective transcripts. (D) Histogram of the correlation coefficient between relative protein levels and their corresponding (relative) transcripts. This is a measure of how proportional proteins are to their respective RNAs. (E) 2-D histogram of the correlation coefficient of protein vs. RNA (on the x-axis) and the protein vs. the time integral of RNA (y-axis). Darker colors indicate more genes in that given bin. There is a strong anti-correlation between the two measures of dynamic correlation indicating that these two quantities are largely mutually exclusive. (F) The correlation between all RNA and protein levels for a single time point is weak (Spearman correlation coefficient of 0.59).

**Figure 3. RNA fractions.**

For each time point the fraction of total RNA that is either non-coding (purple), tRNA (orange), rRNA (green), or mRNA (red). A) RNA fractions for non-ribosomal RNA depleted samples. B) RNA fractions for ribosomal depleted fractions. Each bar represents an individual biological repeat.

**Figure 4. Intra operon correlation.**

(A) and (B) histograms of the median pairwise correlation coefficient between all possible pairs of RNA and Protein profiles, respectively, within an operon. Proteins that have a smaller inter-gene distance are more likely to have correlated profiles (C). 2D Histogram of the pairwise correlation between proteins in the same operon, y-axis, and the inter-gene distance between the protein coding regions, x-axis. (D) and (E) Examples of proteins in the same operon that are highly correlated. (F) and (G) Examples of RNAs in the same operon that are highly correlated.

**Figure 5. Analysis of RNA and protein profiles for long-term glucose starvation**

We group RNA, and protein, time courses based on general qualitative behaviors. In response to glucose starvation RNA can be shut off, turned on, pulsed up, and pulsed down. (A) To sort the profiles, a piecewise continuous curve is fit to our data. The parameter, *t*0, is the time at which we start to collect data at 3hrs into growth. The curve was fit using a differential evolution fitting algorithm that is gradient free and population based, allowing for a range of possible parameter sets that can explain our data given the experimental error. (B)-(F) Four random examples of measured RNA time courses averaged across 3 biological replicates (green circles) with their standard deviations (green bars) along with the corresponding fits (blue). The blue bars represent the standard deviation of the range of fits that agree with our data. Both experimental time courses and fits are normalized by the average of the time course. (G) Most of the RNA’s begin to change between 6-8 hrs, when the cells begin to enter stationary phase. This is demonstrated by the histogram of *t*1, the time to the first inflection point.

**Figure 6. Term enrichment and general behaviors.**

Fitting the mRNA and protein profiles allowed us to estimate the underlying dynamics and differential regulation of each gene sorting them into high confidence bins describing their behavior. Genes where put into bins based upon if they where up-regulated, down-regulated, temporarily up-regulated, or temporarily down-regulated. The mRNA, or proteins, in each bin where then tested for enrichment of GO terms. (A) and (B) shows the average of the mRNAs in a given enriched term for mRNAs that are down and up-regulated respectively. (C) and (D) shows the average of the proteins in a given enriched term for proteins that are down and up-regulated respectively. There was no significant enrichment for either proteins or mRNAs that where temporarily up or down-regulated. As a complementary approach we took the average of all proteins in a given Kegg pathway annotation. (E) and (F) show the average of the proteins in the Kegg pathway that are changing significantly. All the other terms where flat.

**Figure 7. Flux ratio profiles in long-term glucose growth.**

Flux ratios where computed using fiat flux software from GC-MS derived 13C constraints. Only one independent flux ratio showed a significant change over the course of the experiment. Flux ratios for (A) SER from GLY, (B) OYR from MAL upper branch, (C) PEP through TK upper branch, (D) PEP through PPP upper branch, (E) PEP from OAA, (F) OAA from PEP, (G) P5P from G6P lower branch, (H) E4P through TK, and (I) GLY through serine. P5P from G6P lower branch has a large correlation (~0.85) with the corresponding ratio of proteins that catalyze those reactions while the corresponding mRNA ratio shows a much lower correlation (~0.5) with the flux ratios.

**Figure 8. Lipid A and phospholipid profiles in long term glucose growth.**

Lipid A and phospholipids were extracted from all samples for analysis by negative ion MALDI-TOF and ESI-MS mass spectrometry, respectively, and the 6 hour and 2 week representative samples are shown in this figure. (A) Activation of the acyl-transferase PagP, adds a C16 chain to lipid A on the 2-position primary acyl chain, resulting in a m/z of ~2035. (B) Modification of phospholipids by cyclopropanation of one unsaturated double bond is catalyzed by CFA synthase. Transcripts of CFA synthase increase at late times (green) consistent with modification of PE where as the level of CFA protein stays relatively flat at late time points. (C) As represented here by the 6 hr sample, lipid A from all samples collected between 3 and 48 hrs contained one major peak at ~1797 m/z corresponding to wild type, hexa-acylated lipid A. As illustrated on the right by the 2 week sample, the 1 and 2 week (168 and 336 hr) time points show the addition of the C16 chain to lipid A. (D) Phosphatidylethanolamine (PE) is shown here as a representation of similar results obtained in phosphatidylglycerol (PG). The phospholipid profiles of the samples remained relatively consistent with wild type *E. coli* phospholipid profiles until hour 8, when a gradual increase in a peak ~702.5 m/z began and became the predominant species by 2 weeks. This mass corresponds to the cyclopropanation of one unsaturated double bond within a PE molecule containing acyl chains totaling 33 carbons distributed between the two acyl chains.

**Figure 1 - supplemental figure 1.** **Correlations of raw protein and mRNA counts between different biological replicates.**

Scatter plot between biological replicates 1 and 2 (A, C) and 1 and 3 (B, D) along with their associated Spearman correlation coefficients.

**Figure 2 - supplemental figure 1. Correlations between the absolute abundance between protein and mRNA for all time points.**

Both the RNA and protein levels are scaled to their respective averages across all RNAs or proteins for each time point and then log transformed. All P values are <10-46.

**Figure 5 - supplemental figure 1. Distributions of time scales found by fitting the piecewise continuous curve, described in the main text, to the mRNA and protein profiles.**

(A-C) mRNA distributions of *t*1, time to first inflection, the time between the first inflection and the time it takes to stop to changing, and the total time it takes for a given profile to stop changing.

# Supplemental Text

## Consistency of biological repeats

To demonstrate the repeatability of our proteomic and RNAseq pipelines we investigated the correlation between individual biological repeats. To this end, we calculated the spearman correlation coefficient, and plotted the scatter, between the first and second and the first and third biological repeats for both protein and RNA raw counts (Figure 1-supplemental figure 1). Biological repeats show ~0.93± 0.02 <P value is so low as to be 0> correlation in the raw counts for both protein and RNA. Both RNAseq and mass spectrometry where comparably repeatable. Additionally the proteins ID by our mass spec pipeline in a given biological repeat are highly consistent. Just over 2800 proteins are identified across all three biological replicates, with ~3200 protein IDs for any given individual experiment (Figure 1-supplemental figure 2). This is an improvement over the previous record of ~2600 *E. coli* protein IDs in a mass spec experiment <Krug 2012>.

## RNA composition

The overall pool of mRNA decreases over the time course as demands for new RNA and protein is lessened in stationary phase. To visualize this we plot the mRNA, tRNA, and non-coding (NC) RNA, as a fraction of the total RNA (excluding ribosomal RNA) for a given time point(Figure 1-supplemental figure 3). Ribosomal RNA is excluded from our analysis as it is depleted in our RNA prep. In early time points the RNA pool is dominated by mRNA. For late time points tRNAs dominate the total RNA pool.

## Absolute Correlations

Absolute correlation between proteins and their corresponding transcripts are relatively strong for time points <=8hrs with a correlation coefficient of ~0.59. After 8hrs, when cells have entered stationary phase, the correlation is much weaker. At 48 hrs the correlation is the weakest (0.29). In *E. coli* mRNAs typically have relatively fast degradation rates with ½ lives around 5-10min <ref> while proteins half-lives are typically much longer (>30min). During entry into stationary phase mRNA expression is differentially regulated before the 10 hr mark, for the most part, protein levels however take a while to catch up, due to there slower degradation rate. We see this effect in the fact that correlation increases as time goes on after the initial decrease at 24-48 hrs ending up with a correlation of 0.39.

## Distributions of the mRNA and protein profile timescales

Here we further investigate the range of responses to long-term glucose starvation seen in our RNA and protein expression experiments. To do this we plot histograms of the time scale parameters we found by fitting the piecewise continuous curve to our data (Figure 4-supplemental figure 1). The most informative time scales are *t*1, the time to first inflection, and *t*2+*t*3+*t*4 the time it takes for the profile to stop changing. The majority of proteins and their transcripts begin changing before the 10 hr mark (or just after the cells enter stationary phase). Once the profiles begin to change it takes >10 hrs before it stops changing again. However, the apparent long time scale of proteins and transcripts changing could be due to the low time resolution of our experiment after the cells have entered stationary phase.