**Controlled Measurements of Multiple Cellular Components in E. coli As a Resource for Integrative Computational Modeling of Cellular Subsystems**

**John R. Houser, Craig Barnhart, Dan Boutz, Sean Carroll,Josh Michener, Brittany Needham, Ophelia Papoulas, Viswanadham Sridhara, Dariya Sydykova, Christopher J. Marx , Steven Trent, Jeff Barrick, Claus Wilke, Edward Marcotte**

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**

A goal of systems biology has been to understand how phenotype originates from geneotype. 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. Though computational models of individual component subsystems, such as flux models of metabolism1–3, have enjoyed a long history of success, they are still 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 networks4–12 with other studies focusing on integrative, whole-cell, models13,14. Given the growing interest in integrative modeling approaches there is a pressing need for high quality genome-wide data that is comparable across cellular subsystems.

Here we present a long-term glucose starvation experiment with data on genome-wide RNA and protein levels as well as metabolic flux and lipid modification 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 looking up responses to specific genes and/or molecules across cellular subsystems.

To demonstrate our data sets usefulness in the following sections report the results of our in-depth characterization. To characterize the data we compared and contrasted differences between mRNA and protein expression profiles, differences between expression profiles within an operon, differences between metabolic flux and gene expression, and differences between lipid modification and gene expression.

**Controlled measurements of multiple cellular components**

In this section we outline our approach to measure multiple cellular components under identically controlled experimental conditions. We grew multiple cultures, from the same stock, under identical growth conditions of long-term glucose starvation, in the same media, and the cultures were distributed to the different labs to measure RNA, protein, lipids, and metabolic flux ratios (Figure 1A). Freezer stocks of the *E. coli REL606* strain 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-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). Below we characterize our data outlining major trends and comparing and contrasting the response of different cellular subsystems.

**General trends of RNA and Protein time course**

Gene expression is often differentially regulated in order to cope with a changing environment. Due to translational and post-translational regulation we expected differences in the response of proteins and their transcripts 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 analysis we focus on only those RNAs and proteins that are changing significantly (as measured by fdr and fold change cutoff respectively) throughout the time course yielding ~1900 significantly changing transcripts/proteins. In *K*-means clustering it is up to the user to pick the number of clusters that is appropriate such that the profiles are well separated into groups with unique and distinct behaviors. 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. 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.

It is important to note that the above clustering of the RNA and protein 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 transcripts9 . 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 it's transcript. To put proteins and RNA, within a given sample, on comparable scales the protein counts were normalized using the apex method15 for absolute quantification and the mRNA was normalized to the length of each transcript. Both protein and mRNA levels were 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 and it's transcript we had to account for the underlying dynamics of the time courses by considering 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*=…). This number is in-line with measurements of correlations between RNA and protein for other organisms9,16–19. 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.

**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 operon20–23. 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 operon23. We expected 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 3A). On the other hand less than fourteen percent of proteins had a mean pairwise correlation coefficient greater than 0.8 within an operon(figure 3B). Genes closer together within an operon are more likely to have correlated protein profiles (see figure 3C) 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 3 E and C/D respectively). 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 4A). 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 2B-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 4B-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 4F shows the distribution of *t*1, the time to first inflection. Most of the mRNAs respond between 3-8 hrs, during the transition between exponential to stationary growth. 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 5A, B.

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 5D shows the average of the proteins in a given GO term that are enriched in the set of proteins that are being up regulated. 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 stabilities (figure 5C).

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 5E and F depending on if they were down or up regulated, respectively.

**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 6G). 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 6A-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 high correlation with its corresponding protein ratio (ρ=0.85, *P*=…) and low correlation between its corresponding mRNA ratio (ρ=0.5, *P*=…).

**Lipid profiles**

Using negative ion MALDI-TOF and ESI mass spectrometry we analyzed Lipid A and phospholipids, respectively. Around 8hr the ms peaks associated with an addition of the C16 chain to lipid A and the presence of a ~702.5 m/z corresponding to cyclopropanation of one unsaturated double bond within a PE molecule containing acyl chains increased. Both modifications to lipid A and phospholipid continued to increase up to the 2 week period with the 702.5 m/z peak associated with phospholipid modification dominating, by 2 weeks, over the basal major peak at ~688.5 m/z. The enzymes relevant to the above lipidA and phospholipid modifications are pagP and cycloproponated fatty acid synthase (cfa), respectively. PagP transcript levels do not change significantly during the time course, neither did PhoP levels that drive transcription of PagP. Neither PagP or PhoP was observed at the protein level. CFA synthase protein levels increase between 3-6 hrs before decreasing again and CFA synthase RNA levels increase again around 1 week (figure 7B).

**Discussion**

Our data set is a rich resource for comparing and contrasting the response of multiple cellular subsystems. It is well known that mRNA and protein levels do not strongly correlate in an absolute or relative sense. This is a fact clearly demonstrated by our data. Thus it is crucial to have data on both RNA and protein . This gives us information on both transcriptional regulation, as well as post-transcriptional/translational regulation.

We also saw a late modification of Lipids and phospholipids. These modifications are associated with acid resistance in starved cells. Additionally the modification doesn't really follow CFA synthatase levels this potentially highlights the fact that activation doesn't necessarily follow abundance measurements.

**Methods**

**Growth procedure**

*E. coli 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 method24. 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 25. Prior to mapping, all of the single-end reads have had the adapters trimmed using flexbar 2.3126. Mapping was carried out using bowtie2 2.1.0. with –k 1 option to achieve one unique mapping location per read27. Raw number of reads mapping per gene were counted using htseq-count 0.6.028. The exact pipeline is available at https://github.com/clauswilke/Ecoli\_RNAseq/blob/master/src/bowtie\_commands.sh.

**Proteomics**

Forzen 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 al29. 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.

**Lipid analysis**

Lipid A and phospholipids were isolated from bacterial pellets containing 3-9 x 10^9 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 described30. Phospholipids in the supernatant were further purified by extractions as previously described31. 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 described30. Phospholipid analysis was performed by liquid chromatography/ESI-mass spectrometry at Duke University as previously described32

**Data analysis**

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