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Resource allocation in starving bacteria:
proteolysis, protein synthesis, and survival

A Dissertation submitted in partial satisfaction of the requirements
for the degree Doctor of Philosophy

in

Biology with a Specialization in
Quantitative Biology

by

Gabriel Mullin-Manzanarez

Committee in charge:

Professor Terence Hwa, Chair

Professor Eric Bennett

Professor Jeff Hasty

Professor Jim Wilhelm

2024

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University of California San Diego

2024

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DEDICATION

To my parents and brother.

EPIGRAPH

It is not down on any map; true places never are.

Herman Melville, Moby Dick

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I pursued a PhD in microbial physiology to learn more about the cell; what it cares about and how it organizes its many processes. I knew studying quantitative physiology in a physics lab would be a challenging and engaging departure from my previous work in synthetic biology. My first project in the lab was a dive into the physiology of potassium starvation in *E. coli*. Even as a synthetic biologist I had never considered how fundamental the potassium ion is to all living systems (even in the saltiest water where sodium is abundant, organisms still use potassium for fundamental processes such as charge balance in ribosomes). Attempting to uncover meaning through simple, elegant experiments and to deepen our understanding of a fundamental component of many physiological processes was a humbling but rewarding start to my PhD. I felt closer to the living world. And that was just potassium! I am leaving this period of my life with a deeper appreciation of the complexity of life. And a deeper understanding that good things take time.

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PUBLICATIONS

G. Manzanarez[†], R. Balakrishnan[†], H. Kannan[†], M. Abele, Z. Zhang, C. Wu., L. Freiman, M. Mori, C. Ludwig, M*. Mori, T. Hwa*. YeaG-mediated global proteolysis in starving bacteria dictates protein synthesis and prolongs cell survival. *In Preparation*

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*These authors contributed equally to this work.

ABSTRACT OF THE DISSERTATION

Resource allocation in starving bacteria:
proteolysis, protein synthesis, and survival

by

Gabriel Mullin-Manzanarez

Doctor of Philosophy in Biology with a Specialization in
Quantitative Biology

University of California San Diego, 2024

Professor Terence Hwa, Chair

Survival through periods of starvation is crucial for bacterial fitness. The role of gene expression in sustaining the viability of starving bacteria has been extensively studied, including the identification and investigation of 'stress response' proteins regulated by the general stress sigma factor RpoS in *E. coli*. However, less is known about the costs and benefits of metabolic processes sustaining gene expression during starvation. This thesis work describes a comprehensive physiological study of *E. coli* cells under glucose starvation, quantifying the temporal dynamics of resource generation and utilization, and connecting them to cell viability. We focus on a preparatory period, the stationary phase, where the proteome is remodeled after entering starvation, before the onset of significant

population death. We find two proteins are degraded for every protein synthesized, and five-six proteins degraded for every protein accumulated, resulting in the turnover of over half of the proteome for a moderate gain in stress proteins. Such extensive global proteolysis is enabled by a little-known member of the RpoS regulon, YeaG, which works in concert with well-known members of the proteolytic machineries. The total amount of protein synthesis is dictated by the amount of protein degradation, reflecting a dominant, supply-driven strategy of resource allocation for starving cells. YeaG-mediated proteome remodeling accounts for a major share of the viability boost provided by RpoS. In a mutant where energy can be supplemented without cell growth, YeaG activity is largely unnecessary, suggesting that a major physiological function of proteome remodeling is to increase the energy efficiency of starving cells for survival. Such knowledge of the physiology and molecular biology of starving bacteria offers new insights on bacterial vulnerabilities and inspires new strategies for engineering efficient bacterial bio-factories.

Chapter 1: Introduction and Background

1.1 Introduction

The ability for microbes to grow when possible and survive when growth is not possible determines their fitness¹. Growth of any organism relies on an accommodating environment and, as unicellular organisms, microbes are particularly susceptible to the whims of their environment. They often experience rapid and dramatic environmental changes, such as the depletion of key nutrients, changes in temperature, pH, osmolarity, or the presence of toxins, and must fend for themselves². Indeed, it is not uncommon for microbial metabolism itself to alter the extracellular environment during growth, either by the consumption of key resources or by the excretion of potentially toxic metabolic byproducts such as short chain fatty acids³.

Across generations, microbial populations evolve in response to environmental shifts through genetic mutations and natural selection⁴. Additionally, microbes have evolved physiological, non-mutation-based programs in which they coordinate their molecular components to respond to rapid changes in their environment⁵⁻⁹. This work focuses on the bacteria's physiological response to environmental nutrient depletion so extreme that growth is no longer possible: in other words, to nutrient starvation.

Many bacteria, including *E. coli*, are immensely capable of growing quickly in favorable conditions. Indeed at 37°C, in an aerated and nutrient-rich broth, an *E. coli* cell can replicate itself every ~20 minutes, leading to exponential population growth that would overtake the mass of the earth in a few days given infinite resources^{10,11}. Since bacteria are

abundant but have not consumed all global resources (yet), most of the estimated $\sim 5 \times 10^{30}$ bacterial populations on our planet are assumed to be in slow or non-growing states^{11,12}, often due to the lack of available key nutrients for growth¹³. Measuring microbial population growth dynamics and the fractional composition of growth within populations under natural conditions is challenging. However, in studies of native soils as an exemplary ecosystem, less than $\sim 50\%$ and sometimes less than 1% of native soil microbes are actively growing at any particular time^{14–17}. The ubiquity of non-growing bacteria in non-growing states demonstrates the importance of their study in the context of ecology and biogeochemical cycles. Other contexts in which non-growth is important include the development of antibiotics, understanding and modulating the human microbiome, and the large-scale commercial culturing of bacteria for molecule production. This work represents a comprehensive approach to the study of microbial physiological response to growth arrest, via the fast-growing bacteria, *Escherichia coli*, and its response to carbon starvation, a severe stress given the fundamental role carbon plays in growth and survival.

1.2 Bacterial fitness in fluctuating environments

A bacterium's fitness is tied to its ability to grow when possible and survive when growth is not possible. Bacteria can control their physiological response to external stressors such as nutrient starvation, which can be repetitive or unpredictable, via gene expression. The ability for cells to evolve physiological responses to the type and frequency of stressors they experience in their ecological niche will determine their fitness in that environment. In the example of nutrient starvation as illustrated in Figure 1.A, the ability for

a bacterial population to survive requires maintaining cell density over cycles of nutrient availability and depletion. Via gene expression programs, bacteria can tune lag time (T_{lag}) and growth rate (λ) when nutrients are introduced as well as time in stationary phase (T_{stat}) and death rate (δ) when one or more key nutrients are not available. Studies indicate that a cell cannot optimize all parameters which leads to tradeoffs between these physiological^{18–20}. For example, growth rate can come at the expense of death rate¹. Or, as will related to this work, degradation of a key anabolic proteins during carbon starvation to enable survival can lead to a longer lag before exponential growth when carbon is reintroduced²¹.

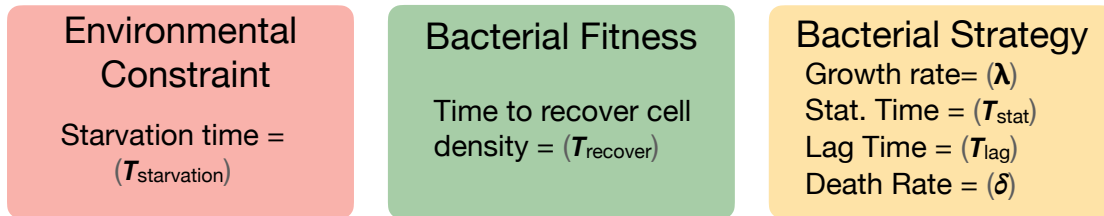
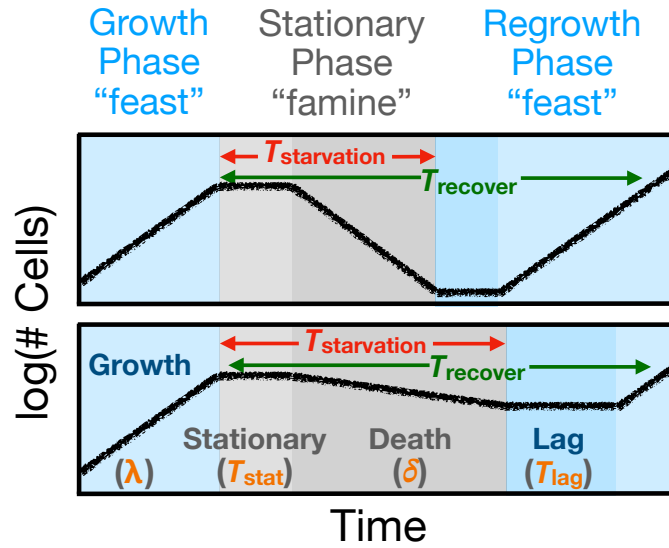


Figure 1.A Illustration of environmental constraints and bacterial physiological parameters in cycles of nutrient availability

Nutrient availability enabling exponential growth will often be intermittent, leading to periods of nutrient availability ($T_{recover}$) and starvation ($T_{starvation}$) of varying durations. Four distinct phases of bacterial population behavior are typically observed^{22–24}: lag, growth, stationary, and death. When nutrients become available, bacteria can evolve gene expression programs to tune their lag time (T_{lag}) before exponential growth as well as their exponential growth rate (λ). When one or more key nutrients are depleted from the environment, bacteria can evolve gene expression programs to tune their time in stationary phase (T_{stat}) and their death rate (δ). The fitness of bacterial populations in an environmental niche will be determined by maintaining population numbers across the specific patterns and durations of nutrient starvation along with surviving other stressors.

1.3 Carbon starvation

Carbon forms the backbone of biomass molecules and the metabolism of carbon molecules is therefore required for novel biosynthesis and cell growth^{25–28}. Additionally, as with all heterotrophs, *E. coli* primarily relies on energy gained from the catabolism of carbon-carbon and carbon-hydrogen bonds to fuel anabolism as well as the fundamental processes required to stay alive, such as maintaining membrane potential^{29–35}. Growth will cease without the availability of exogenous organic carbon molecules, and the catabolism of carbon bonds is essential for maintaining viability. But what determines this maintenance demand and intracellular carbon economy during exogenous carbon starvation? This work attempts to capture key elements of the intracellular carbon economy in a carbon-starved cell population.

1.4 *E. coli* lifestyle and use in commercial applications

The species *Escherichia coli* encompasses a set of gram-negative bacterial strains in the phylum *Proteobacteria*, class *Gammaproteobacteria*, order *Enterobacterales*, and family *Enterobacteriaceae*. *E. coli* is prevalent in the large intestine of various vertebrate species. In humans, it is found in approximately 90% of individuals^{36,37}. *E. coli*, a facultative anaerobe, is the primary non-anaerobic organism in the human gut³⁷. However, in the anaerobic environment of the large intestine, obligate anaerobes outnumber *E. coli* by two to four orders of magnitude³⁸.

In terms of carbon sources for microbial metabolism, the large intestine is a complex mixture of polysaccharides, oligosaccharides, peptides, mucosal glycans, and single chain

fatty acid fermentation products with much of the readily metabolizable carbon sources such as sugars and amino acids absorbed by the host upstream of the large intestine^{39–41}. *E. coli* can metabolize a broad range of carbon molecules, including sugars, sugar alcohols, organic acids, and amino acids^{42–45}, making it adept at scavenging for carbon sources during nutrient-limited periods. Despite the periodic nature of food consumption by the host, studies suggest that metabolizable carbon is usually present in the large intestine, especially since the dietary fiber is the major food source and takes time to breakdown into consumable sugars^{40,41,46}. However, upon expulsion from the host in feces, an *E. coli* cell likely faces periodic carbon starvation in waterways, soils, and plants until it dies or reaches a new host^{16,47–52}. Estimates suggest that at any given time, approximately 50% of *E. coli* cells globally reside in the gut, while the other 50% reside outside the gut, justifying the need for an *E. coli* evolving a physiological program to survive starvation⁴⁷.

While its native lifestyle suggests the importance of surviving carbon starvation, particularly during transit periods between hosts, the prevalent usage of *E. coli* in applied settings where nutrient deprivation can occur is also an important consideration. Laboratory strains of *E. coli* have been extensively studied^{44,53}, particularly during their growth phases, which has led to the development of tools and their use as a workhorse in industry. Approximately 50% of recombinant protein production, in an industry valued at nearly \$400 billion, is carried out using engineered *E. coli* cells^{54,55}. Additionally, exciting progress has been made in engineering *E. coli* cells for more dynamic functions, such as detecting or killing cancer cells in the human body^{56,57} or monitoring and remediating water quality⁵⁸. In

these applied settings, whether in large fermenters, in the human blood stream, or in contaminated water, *E. coli* is likely to experience carbon starvation which can alter the engineered function.

1.5 A historical note about the study of nutrient starvation

While population growth arrest has been identified as a key phase of the bacterial lifecycle for over a century^{5,22-24}, the study of bacteria in non-growing states remains relatively underexplored compared to the bacterial growth phase. This is partly due to the difficulty in defining and coordinating a focused study of non-growing states across research groups, as non-growth can result from various stressors, each triggering distinct physiological responses^{5,59}. Moreover, different bacteria employ a range of strategies to cope with these stressors^{5,60}. Finally, unlike exponential growth, the non-growth state is dynamic and can encompass various phases that range from the stationary phase, which occurs on an hours-long timescale, to long-term dormancy, which can last for years. These phases often involve subtle and challenging-to-detect physiological changes⁶⁰⁻⁶⁵.

Historically, research into nutrient starvation in particular focused on biomass turnover, or the catabolism of a cells own molecules (e.g. protein, RNA, lipids, DNA) to provide ATP and building blocks for anabolism, with early work from the 1950s to the 1980s employing radiolabeling techniques⁶⁶⁻⁷⁰. These methods enabled highly quantitative measurements of biomass turnover under nutrient-limited conditions, but studies were spread across a broad range starvation conditions and bacterial species and an integrated picture of how turnover was coordinated has not been solidified⁵.

In the 1980s, as new experimental methodologies emerged, research on bacterial nutrient starvation shifted towards measuring global gene expression. This shift was driven by the development of two-dimensional gel electrophoresis, and later, advanced techniques like transcriptomics and proteomics to identify gene expression patterns^{13,71,72}. This laid the groundwork for more detailed molecular studies in the 1990s through 2000s, where research began to focus on the regulation and molecular mechanisms of specific gene products^{73–75}. The approach presented in this work bridges these historical perspectives by integrating the study of biomass turnover and protein synthesis, and by identifying a molecular mechanism that links these two key components of bacterial survival.

Experimentally, to study these salient cellular responses to carbon starvation in *E. coli*, we employ classical biochemical and microbial physiology methodologies complemented by contemporary systems biology tools and perspectives. While nutrient starvation in natural settings may occur within microbial communities and involve other environmental stressors including predation, we incur carbon starvation in a controlled environment to study the cell's specific stress response to this stress.

Chapter 2: Resource allocation towards protein synthesis

2.1 Experimental setup: glucose starvation via a defined “natural runout”

To study the temporal dynamics of starving bacteria in a defined, robust manner, we set up exponentially growing cultures of *E. coli* K-12 cells to abruptly run out of glucose, the sole carbon source, at a fixed, intermediate optical density (OD_{600} , a proxy for biomass density)⁷⁶, such that the increase of biomass is halted within a few minutes and the culture remains fully aerobic and replete with all other nutrient components⁷⁷ (Figure 2.Aa, Figure 2.Ba, Chapter 2 Methods). To avoid extensive death and the associated nutrient recycling due to cannibalism⁷⁸ we used MOPS medium in which the death phase is delayed⁷⁹ (Figure 2.Bb). In these conditions, viability decreases minimally for wildtype cells over the next several days (circles, Figure 2.Ab), after an initial rise due to reductive cell division^{80,65,62} (Figure 2.B). Viability drops rapidly in a strain lacking *rpoS* (squares, Figure 2.Bb), which encodes the general stress sigma factor RpoS^{81,82} reflecting the known importance of the RpoS regulon in prolonging the survival of starving bacteria^{81,83,71}. In bacteria, sigma factor, are proteins that bind with RNA polymerase to form a holoenzyme and are required for transcription, with each of *E. coli*'s 7 identified sigma factors promoting transcription of different genes via influencing affinity of the holoenzyme to different sets of promoters⁸⁴. RpoS, in particular, has been demonstrated to promote the transcription of a set of genes that promote fitness under a variety of stress conditions⁸¹.

Figure 2.A: Resource allocation and protein synthesis in starving cells

(a) Culture of *E.coli* strain K-12 NCM3722 (WT)⁸⁵ growing exponentially in glucose minimal medium at mid-log was diluted to a fresh medium with limiting glucose (2 mM); exponential growth continued for several doublings until optical density (OD₆₀₀, top panel) reached ~0.4 when growth was abruptly arrested (vertical dashed line, $t=0$) due to glucose depletion (bottom panel). Unless otherwise indicated, all starvation experiments in this study were performed this way, which is highly repeatable, see Figure 2.Ba, with measurements taken after the culture ran out of glucose at $t=0$. (b) Viability of WT and $\Delta rpoS$ cultures at various times after starvation was characterized by counting colony forming units (CFU) on Luria Bertani (LB) plates (Figure 2.Bb). (c) Illustration of amino acids (AA) and ATP (energy) generation through protein turnover during starvation. Protein degradation releases free AAs, the flux of which is represented by the blue and grey arrows. A portion of these AAs are reincorporated into newly synthesized proteins, others are catabolized to generate ATP (needed to fuel protein synthesis and maintain vital cellular functions). Some non-catabolizable AAs in surplus are excreted (Figure 2.Da). (d) Total protein abundance of WT and $\Delta rpoS$ cultures was measured by radiolabeling (Appendix A1) for procedure and reproducibility). The results were expressed as percentages of the Initial Total Protein Mass (%ITPM), which is the total protein content at the end of exponential growth ($t=0$), 333 μg protein/OD/mL or 616 mg protein/gCDW⁷⁶. 1% ITPM correspond to approximately 40000 copies/cell; see Figure 2.E. (For CFU measurements, the value of each biological replicate is obtained as the average over three technical replicates.) The vertical bars represent standard errors. (e) The estimated upper bound in protein synthesis, assuming that the protein loss measured in (d) is converted completely to ATP, and this ATP obtained is used exclusively to fuel protein synthesis at 4 ATP per peptide bond. Plotted are estimates based on the AA-ATP conversion efficiency given in the legend for WT and $\Delta rpoS$ cells. (f) The expected amount of protein degradation is obtained as the sum of the expected synthesis in (e) and the measured amount of protein loss in (d).

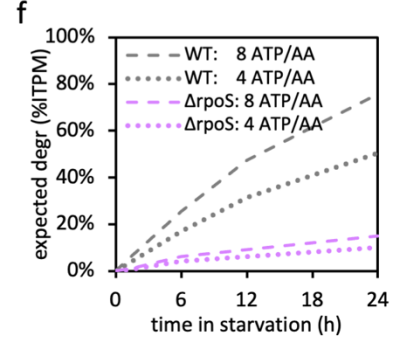
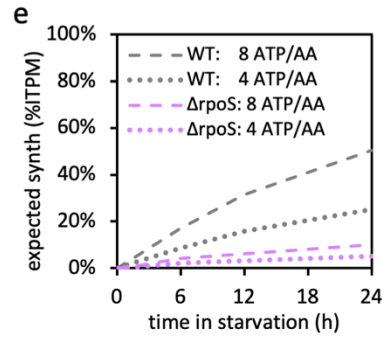
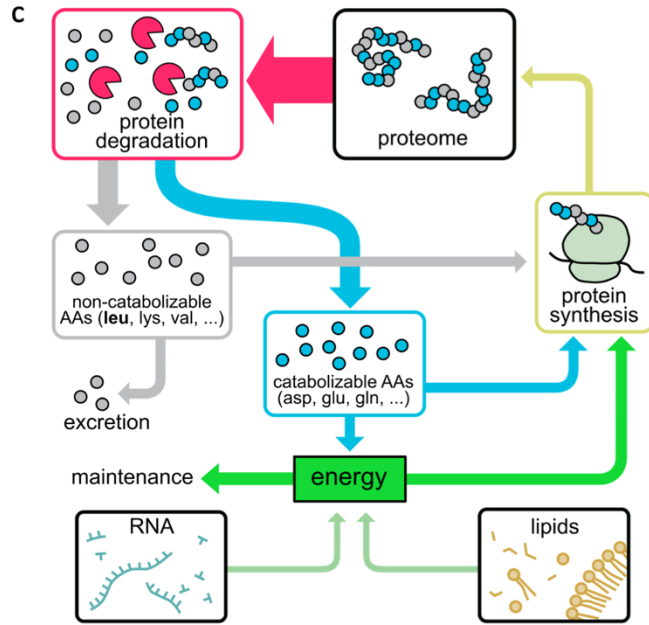
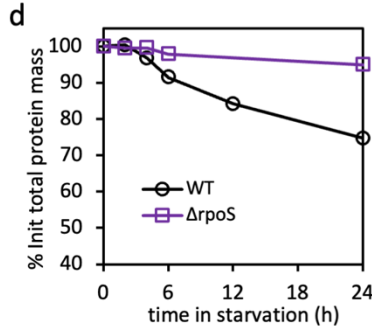
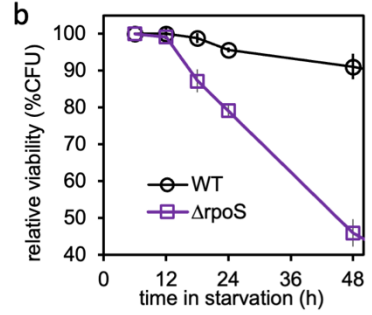
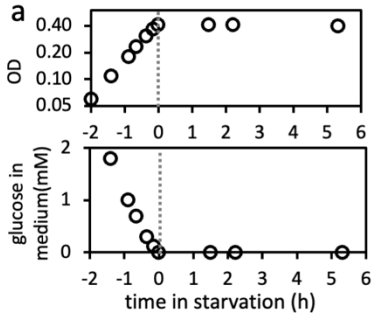


Figure 2.B Growth curves, viability, and reductive division

(a) The behaviors of stationary cells depend on the dynamics of how nutrient depletion sets in. In this study, we apply the “natural runout” protocol (Methods) where glucose is rapidly depleted by a culture of exponentially growing cells (Figure 2.Aa). The course of growth arrest is highly reproducible, in terms of the timing and the OD at runout (at $OD \approx 0.4$ for 2mM glucose), as demonstrated by the plot of OD for four biological replicates. Unless otherwise indicated, all measurements reported in this work pertain to the amount per culture at various times after glucose runout ($t=0$). (b) The number of colony forming units (CFU) per mL of the starving culture, normalized by the OD at the time of glucose depletion ($OD \approx 0.4$), referred to here and elsewhere as “iOD”. The viability curve exhibits a sharp rise during the first several hours of glucose starvation, then declines gradually over the course of the next 10 days. The gradual decline in viability is divided naturally into two phases, the “stationary phase” where viability barely drops, followed by a “death phase” where the viability drops exponentially. The exponential nature of the viability drop in the death phase results from nutrient recycling⁷⁸. This work focuses on what happens during the first 24h of the stationary phase and its impact on the duration of the rest of the stationary phase.

The initial sharp rise of the viability curve is due to the “reductive cell division”, a well-known process by which a sub-population of growth-arrested cells complete the current round of DNA replication and carryout another round of cell division to separate the newly generated chromosomes, thus increasing cell number in the absence of biomass accumulation^{5,80}. In the main text, CFU data is always plotted relative to 6h after glucose depletion to avoid referring to the reductive division. In the remaining panels, we provide some details of the reductive division process: (c) CFU of WT and $\Delta rpoS$ cultures during the initial hours after glucose depletion, exhibiting an approximate doubling of cell number, attributed to reductive cell division. The two cultures grow similarly and have similar number of cells/OD during exponential growth, reflecting the fact that RpoS is hardly expressed during exponential growth on glucose⁸⁶. The data here shows that these two strains are also similar in the early hours of glucose starvation. (d,e) Microscopy image of wild-type cells at the moment before growth arrest i.e., 0 hours (panel d) and at 6 hours (panel e). Scale bars indicate 5 μm (f,g). Histograms of cell lengths (panel f) and widths (panel g) at 0 h (black line) and 6 h (grey line) after glucose depletion for wild-type cells. (h,i) Average cell length (panel h) and average cell width (panel i) of wild type cells (black circles) and $\Delta rpoS$ (purple squares) at various times after glucose depletion. For panels (f)-(i), approximately 200 individual cells for each timepoint and each strain.

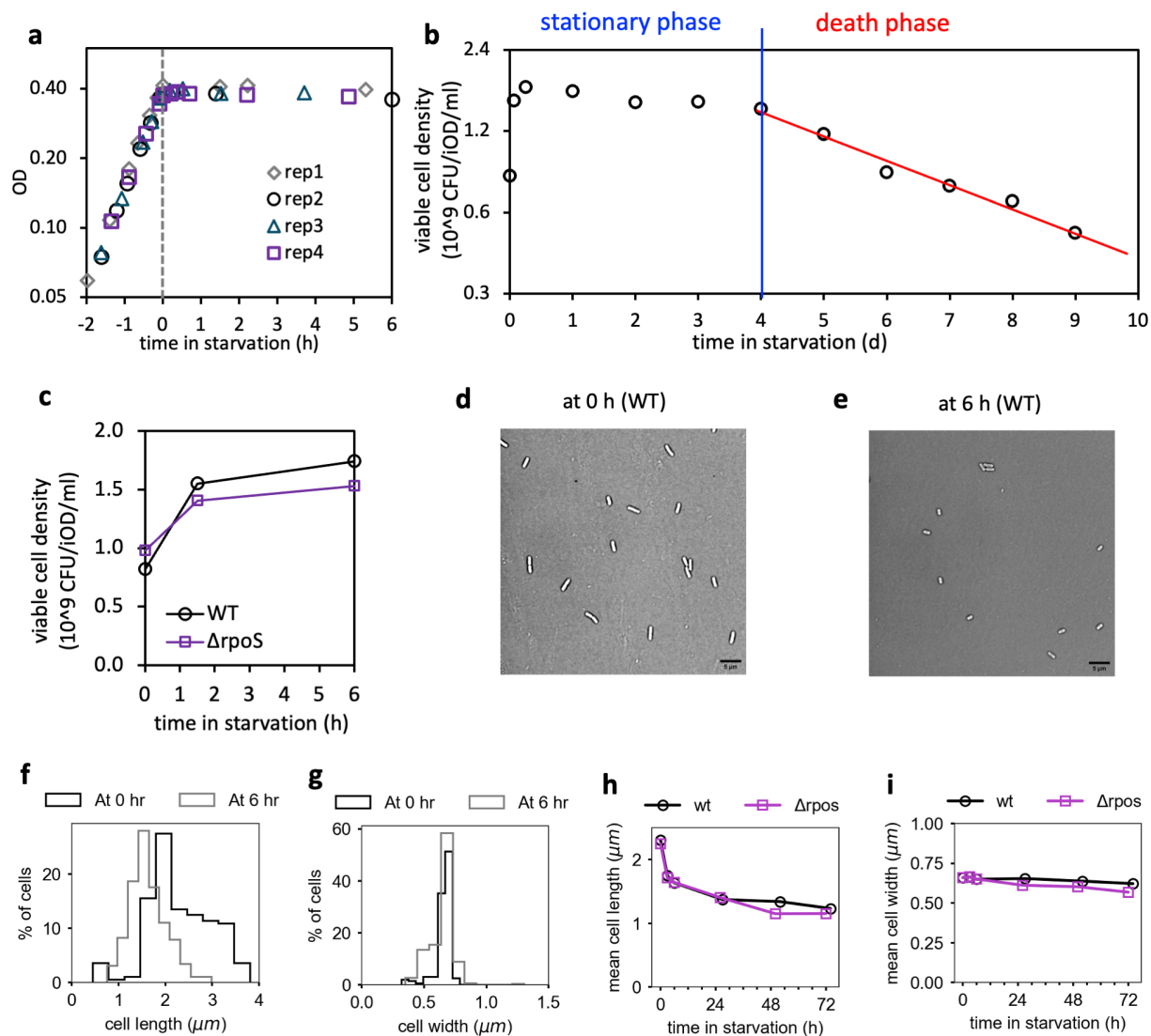


Table 2.1: Protein quantification and ITPM

As shown in Figure 2.Ad, we quantified protein abundance in starved cells as fraction of the initial total protein mass (fITPM) which provided the absolute amount of a protein referenced to the total protein mass at the onset of starvation. This definition differs from that of protein mass fractions since the latter are defined as the mass of a protein as a fraction of the total cellular mass in the same condition or sample. Thanks to its definition, variations of $fITPM_i(t)$ over time reflect genuine changes in cellular protein abundances over time; on the other hand, protein mass fractions are affected by changes in the total protein content of the cell and are thus less suitable for protein quantification outside of exponential phase.

Conversion of protein mass fractions to cellular concentrations or copy numbers have been described extensively in Ref. [87]. Unlike exponential phase, the transition to starvation conditions on which we focus in this work is characterized by an initial increase in copy number without biomass accumulation due to reductive division, followed by an approximately constant cell copy number for a few hours before the onset of the death phase (Figure 2.Ba-b). Thus, changes in $fITPM_i(t)$ match corresponding changes in protein copy numbers, as long as the number of viable cells remains constant. Using the measured total protein abundance at the onset of starvation, 333 ug/ODmL^{76} and the measured CFU (Figure 2.Bb), we can convert $fITPM_i$ into mass of the i^{th} protein per cell, which in turn can be converted to protein copy number per cell. For a representative protein 250 residues in size, and using $1.76 \cdot 10^9 \text{ CFU/ODmL}$ (the value 6h into starvation, after reductive division) we obtain the following copy numbers:

Fraction of ITPM	#proteins/cell
0.01%	414
0.1%	4,140
1%	41,400

For proteins of different sizes, the conversion to copy numbers is changed in inverse proportion to the mass of the protein, see examples below for a protein at 0.1% ITPM:

Protein size	#proteins/cell
125 aa	8,280
250 aa	4,140
500 aa	2,070

2.2 Constraints on protein synthesis during starvation

Protein synthesis requires amino acids (AA) as substrates and ATP as fuel. To understand how these resources are derived and utilized during starvation, we focus on the first 24 hour after glucose depletion where most of the gene expression takes place⁸⁸. Since the viability of WT cells is largely maintained during this period (Figure 2.Ab), nutrient recycling from dead cells in the culture⁷⁸ is negligible and resources must be recycled internally within individual starving cells. Recycling of AAs from protein turnover provides in principle an endless source of AAs. *De novo* AA biosynthesis from recycling other biomass components can also occur; however, quantitative estimates indicate that the capacities of these processes are limited.

2.3 Sources of amino acids for protein synthesis during starvation

Upon the abrupt stoppage of carbon influx (Figure 2.Aa), *E. coli* continues to synthesize proteins for many hours⁷¹. A large portion of the AAs needed for protein synthesis is derived from the turnover of existing proteins as this study will establish. Here we discuss other sources to derive AAs.

Glycogen is *E. coli*'s main carbon storage mechanism. However, glycogen comprises of only 2.5% of the *E. coli* cell dry weight (CDW) and has been previously found to be consumed within 10 min of starvation⁸⁹. RNA is the 2nd most abundant macromolecule in *E. coli* after proteins, comprising of ~18% CDW for cells growing exponentially on glucose⁹⁰ (Table 2.2). However, the conversion of RNA to AA is carbon-inefficient, since only the ribose backbone of the RNA can be catabolized by *E. coli*⁹¹. With the average molecular weight of

nucleotides being 340, there is 0.53 mmol ribose/gCDW. Assuming that all ribose molecules are converted to AA 1-to-1, then the cellular RNA could potentially make 0.53 mmol AA/gCDW. This is equivalent to only 9.5% of proteins, since with 62% of CDW being protein and with the average molecular weight of an AA being 110, the amount of AA fixed in proteins is 5.6 mmol/gCDW. Given that ribosomal proteins rely on binding to rRNA for stability, conversion of RNA to proteins is costly.

Other biomass components such as lipids and osmolytes (mostly glutamate) cannot make the full set of 20 AAs. Acetyl-coA, derived from the turnover of lipids, can be used to synthesize TCA intermediates, which drives the synthesis of 13 “lower” amino acids: asp and 5 AAs derived from asp (asn, lys, met, thr, ile), glu and 3 AAs derived from glu (gln, prol, arg), and ala, leu, val derived from pyr⁴⁴. But gluconeogenesis is required to make the remaining 7 “upper” amino acids: ser, gly, cys requires the synthesis of pep from TCA intermediates or pyr, and trp, tyr, phe, his requires ribose or f6p. It was previously shown⁹⁰ that even with a large amount of acetate available externally, it took *E. coli* many hours to synthesize the enzymes of gluconeogenesis to make the full set of AAs.

In principle, it is possible to use the turnover of RNA to synthesize the upper AAs only, while using lipids and glutamate to synthesize the lower AAs. For example, the amount of glutamate as an osmolyte is 0.1 mmol/gCDW³. If this amount is used to synthesize the 13 lower AAs, and 0.05 mmol/gCDW of ribose (from 10% RNA turnover) is used to synthesize the 7 upper AAs, then (assuming an approximate 1-to-1 conversion) this can produce 0.15 mmol/gCDW of a complete set of AAs, thus enabling the synthesis of 2.7% of proteins. As

we establish in this work, this is a very low AA yield compared to the AA flux that *E. coli* can generate from global proteolysis.

Table 2.2: Calculation of energy yield of biomass components

The table summarizes the maximum energy content from each biomass component. The biomass composition was taken from Ref. [92], and the stoichiometric ratios of each components (amino acids, nucleotides, lipids) in each macromolecule were used to calculate the ATP yield per macromolecule mass (columns under “ATP yield”; unit is mmol ATP per g of the corresponding macromolecule). The product of ATP yield and biomass abundance yields the total energy content per gram of cell dry weight (CDW). The energy yields and content depend on the efficiency of the electron transport chain; we report here the yields obtained in four different cases, as explained in Table 2.1. The DNA energy content is not available in practice as it is not possible to degrade DNA without losing viability.

Biomass group	Fraction of dry mass (mg/gCDW)	ATP yield (mmol/g)				Energy content (mmol ATP/gCDW)			
		WT	Δ nuo	Δ cyo	$\frac{\Delta$ nuo Δ cyo	WT	Δ nuo	Δ cyo	$\frac{\Delta$ nuo Δ cyo
RNA	177	73.0	56.7	55.4	39.1	12.9	10.0	9.8	6.9
Protein	619	71.2	56.5	51.8	37.2	44.1	35.0	32.1	23.0
DNA	23	78.5	59.6	58.9	40.0	1.8	1.3	1.3	0.9
Cell wall - FA	39	213.2	149.5	157.4	93.7	8.2	5.8	6.1	3.6
Cell wall - PG	27	121.5	93.9	88.8	61.2	3.3	2.6	2.4	1.7
Cell wall - LPS	36	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Others - Glycogen	26	151.1	117.2	114.1	80.2	3.9	3.0	2.9	2.1
Others - Glutamate	30	114.6	92.4	83.8	61.6	3.4	2.8	2.5	1.8
Others - Glutathione	22	98.8	76.5	70.2	47.9	2.2	1.7	1.5	1.1
Others - Remainder	2	NA	NA	NA	NA	0.0	0.0	0.0	0.0
Sum	1000	NA	NA	NA	NA	79.8	62.2	58.7	41.1

2.4 Energy content of biomass components

Deriving energy from biomass components is more challenging. Some AAs derived from protein turnover can be catabolized to generate energy (Figure 2.C). Non-catabolizable AAs are excreted into the medium⁹³ (Figure 2.D) as reflected in Figure 2.Ac, where protein loss was measured to reach ~25% of the initial total protein mass (ITPM) by 24h of glucose depletion (circles, Figure 2.Ad, Appendix A1 and A2, Table 2.1). Using flux-balance analysis⁹² (FBA), we obtained a maximal conversion efficiency of ~8 ATP per AA on average (Figure 2.D). Thus, to generate the required energy needed for protein synthesis, at 4 ATPs per peptide bond⁹⁴, on average an extra one-half of a protein needs to be degraded for every protein synthesized, a 50% energy surcharge if energy is derived completely from AA catabolism.

Additional energy can be obtained from the catabolism of other biomass components, particularly from RNA and lipids^{62,68,89,95} (Figure 2.E) Their smaller biomass contents along with their requirements for cellular function and integrity limit the amount they can contribute towards energy. Given calculations of ATP yield for monomers from FBA (Figure 2.E), ATP derivable from proteins, 44 ATP mmol/gCDW, is about 50% of the total derivable from biomass. ATP derivable from RNA (13 ATP mmol/gCDW) and lipids (14 ATP mmol/gCDW) each comprises about 1/3 of the remaining 50%. The ATP yield per nucleotide residue (~25 ATP per nucleotide) is actually 3x higher than that of an average AA (~8 ATP/AA); however, the biomass composition is such that the number of nucleotides is only ~1/10 of the number of AAs. Lipids have even higher energy content but constitute an even smaller fraction of the biomass (Table 2.2). The energy content of the remaining biomass

components is negligible, and the latter are often rapidly depleted upon entering starvation. For example, glycogen is consumed within 10 min of starvation⁸⁹, while the free glutamate pool, comprising a few percent of the total AA in proteins, is depleted within ~20 min⁹³. Lipids degradation cannot be too extensive, given the necessity to preserve membrane integrity. Thus, the only expandable biomass component other than proteins is RNA. In fact, RNA can be quickly synthesized *de novo* by metabolic enzymes once nutrients return, as long as biosynthetic enzymes are present. However, a major drawback of RNA degradation is that the loss of RNA leads to the loss of ribosome proteins, since several r-proteins are prone to proteolysis without being in the ribosome complex^{96–98}. Thus, the catabolism of RNA for energy has a serious long-term cost, i.e., the loss of r-proteins, which threatens the cells' long-term viability and recovery.

To contribute the amount of energy equivalent to 25% of protein loss, it would take ~80% of the total RNA or lipid content (Figure 2.E), while the measured loss of RNA and lipids in the first 24h are no more than the protein loss (black bars, Figure 2.F). Thus, we focus henceforth on the turnover of proteins as the major supplier of energy.

Figure 2.C: Sources of amino acids for energy to fuel protein synthesis during starvation

Here we summarize the known amino acids degradation pathways, based on the information available in the ecocyc database⁴⁴ and references therein. As shown in the figure above, panel (a), each pathway starts with a catabolizable AA (in blue) and ends on a TCA intermediate, alpha ketoglutarate (akg), succinate (succ), fumarate (fum), pyruvate (pyr), and acetyl coA (ac-CoA), all in magenta. Energy products are shown in red, with QH2 being shorthand for ubiquinol; in some cases, transaminase reactions (marked by GDH) have been accounted for by an additional NAD(P)H. Enzymes catalyzing each reaction are indicated next to each reaction arrow: those listed in on the same line are all needed for the reaction; those stacked vertically form parallel pathways and are replaceable. Reaction arrows are color-coded in either green or grey depending on enzyme levels, with green arrows indicating catabolic reactions for which enzymes are expressed in glucose media (see below). Dashed arrows represent alternate pathways likely not used in exponential phase.

We made use of previously published data for *E. coli*⁹⁹ to classify the enzyme based on their levels during growth on glucose minimal media or on casaminoacids (cAA) in absence of glucose, which requires extensive AA catabolism. Enzymes in green are expressed in glucose minimal media to levels comparable (50% or higher) to those on cAA and are therefore immediately available for AA catabolism at the onset of carbon starvation. TCA enzymes and the pyruvate dehydrogenase complex (PDH), connecting the TCA intermediates (magenta), are all highly expressed, as well as the important enzyme MaeA, connecting pyruvate and malate. The rest of the enzymes is either expressed at low levels in both glucose and cAA media (protein mass fractions below 10⁻⁴, grey boxes), or significantly upregulated in cAA media compared to glucose (grey). In the latter case, such strong upregulation suggests that these enzymes play a catabolic role during growth on cAA, but they must be synthesized when glucose is no longer available as carbon source.

We further detailed in panel (b) two composite pathways, indicated in figure by the numbers j and k. The first pathway shows the degradation of glutamine to glutamate using the GS/GOGAT pathway. Although the conversion of glutamine (gln) to glutamate (glu) can in principle be catalyzed by two glutaminases encoded in the *E. coli* genome, glsA and glsB, they are not expressed. Instead, j involves only enzymes that are abundantly expressed during growth¹⁰⁰. The second pathway shows the possible interconversion of prp-coA to pyruvate using the prp operon, which enables propionate catabolism. However, the low levels of the PrpCDB enzymes in rich media suggest that threonine is degraded via glycine and serine as indicated.

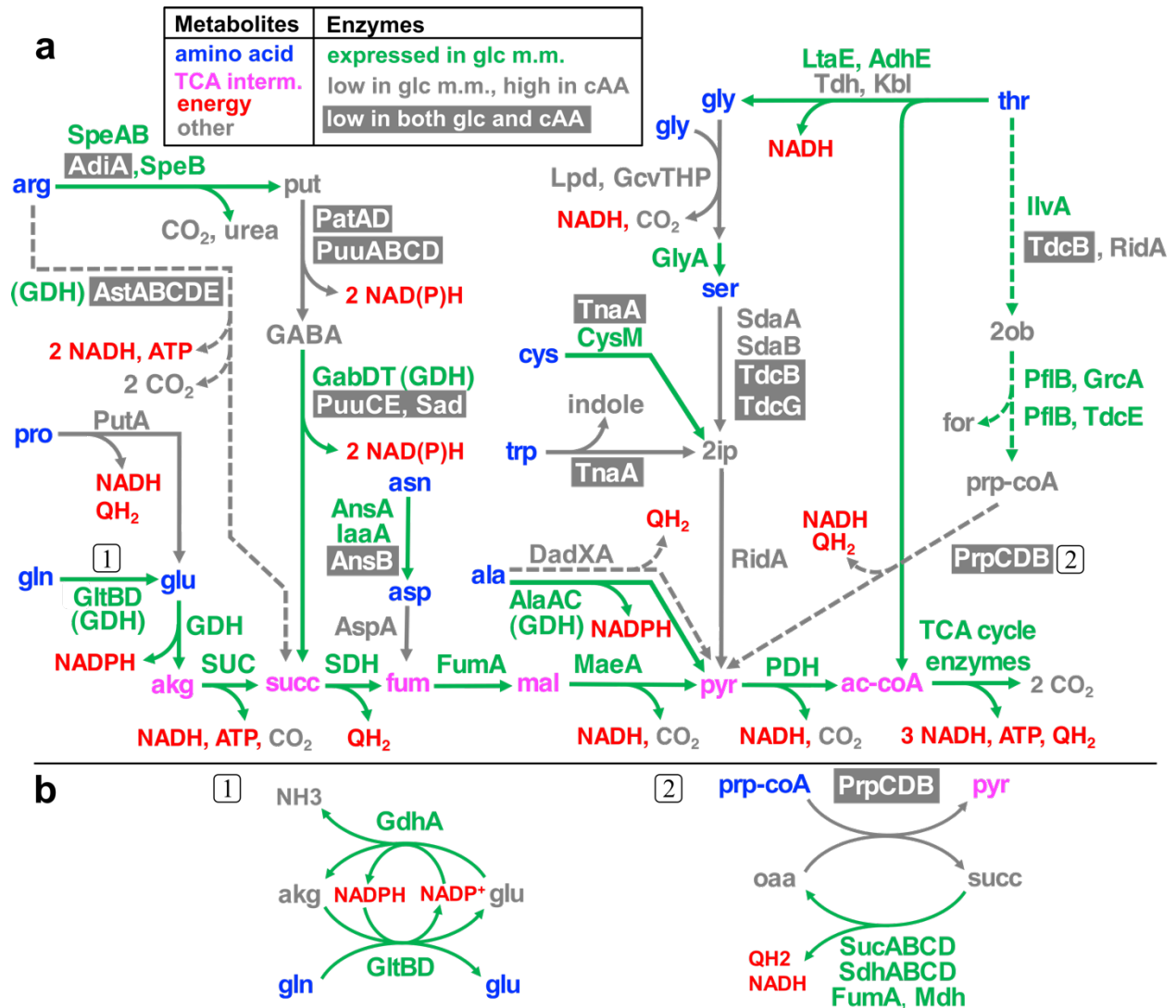


Figure 2.D: Catabolism of amino acids for energy generation

(a) As discussed in Figure 2.C, there are 12 catabolizable AAs; the other 8 AAs are non-catabolizable: leu, ile, val, met, lys, his, tyr, phe. The levels of these AAs rise over the course of 24-h glucose starvation (data reproduced from Ref. [93]), except for lys, whose level turned around after ~5h, suggesting its conversion to other substrates, possibly GABA¹⁰¹. The pool of trp, which is catabolizable, nevertheless accumulates over the first 12h of starvation, suggesting the lack of trp catabolic enzyme, TnaA. The pool of arg rose in the first 1-2 hours⁹³ before dropping gradually downwards, suggesting catabolism after the first hour. (b) The proteomic data collected in this work shows that TnaA and enzymes of the arginine degradation pathway (AstABCDE) accumulated over the first 6h of starvation, corroborating the temporal excretion patterns observed for these two AAs, although differences in strains and conditions allows only a qualitative comparison. (c) The red bars show the number of ATP molecules derivable from each catabolizable AA, obtained from using Flux Balance Analysis with results tabulated in Table 2.3. Trp and Arg, the two catabolizable AAs which accumulated for some time after starvation, are shown as open red bar. The average ATP derivable per AA, weighted by the frequency of that AA in the E. coli expressed proteome (black bars, from Ref. [92], are shown as the horizontal dotted and dashed lines, with Trp and Arg included (7.8 AA/ATP) and excluded (6.6 ATP/AA), respectively; see Table 2.3 for details

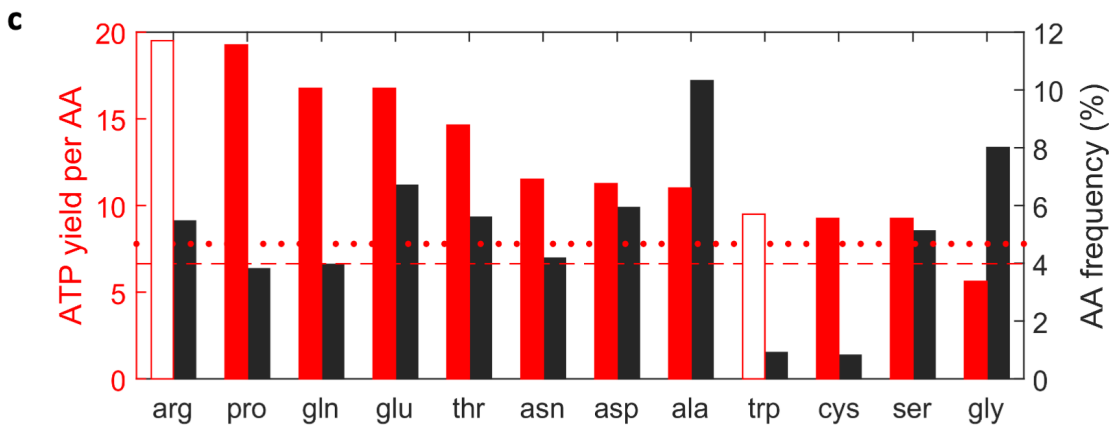
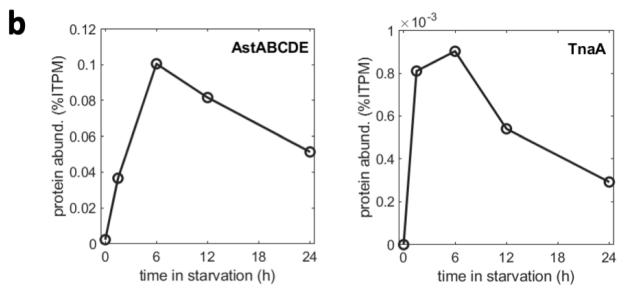
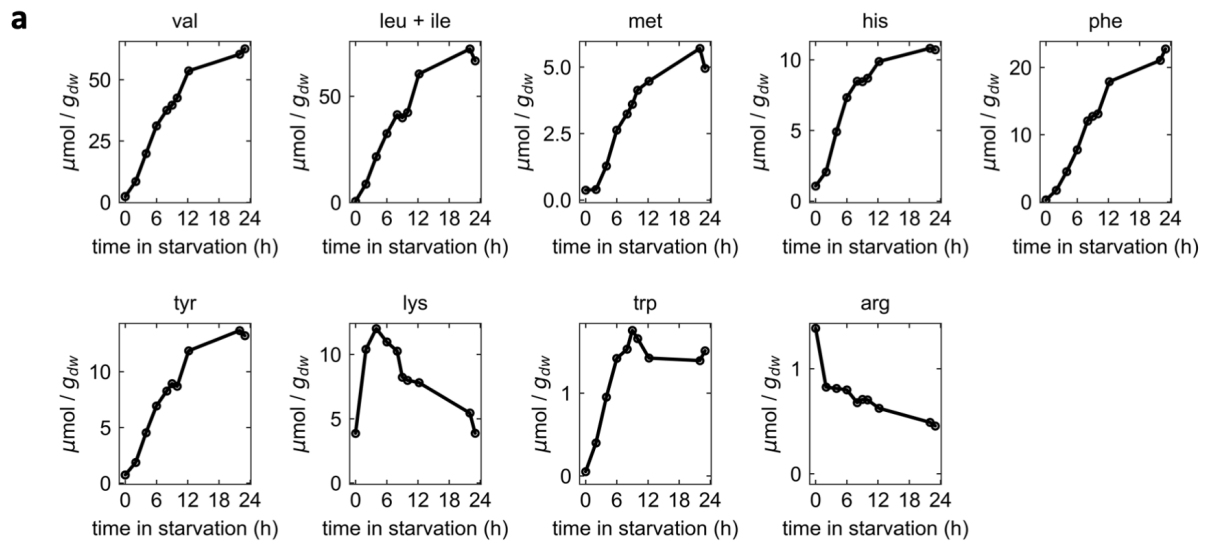


Table 2.3: Calculation of energy yield for individual amino acids

Maximum yields per amino acid were calculated using Flux Balance Analysis (FBA). The aerobic energy yield depends critically on the efficiency of the electron transport chain, which can vary between 1 to 4 protons per electron¹⁰²; this efficiency is likely not maximal for *E. coli* cells grown on glucose^{103, 92}. Thus, we compared yields obtained for the default metabolic model¹⁰⁴ (iML1515) able to use the most efficient conversion factor, to those obtained with *in-silico* mutants lacking either the efficient NADH dehydrogenase NDH-I (encoded by the *nuo* operon), the efficient cytochrome oxidase BO3 (encoded by the *cyo* operon), or both. The average ATP yield per amino acid, weighted by the residue frequency in *E. coli* proteome, varies from about 8 ATP/AA in the most efficient case, to about 4 in the least efficient case. Furthermore, arginine and tryptophan are not used in during the early hours of glucose starvation⁹³, consistent with the low levels of enzymes of Arg degradation encoded by the *ast* operon, and low level of Tryptophanase encoded by *tnaA*; see Figure 2.A. The bottom rows show the numbers recalculated excluding the contribution of these two amino acids.

residue	residue frequency (%)	residue mass (g/mol)	WT	Δnuo	Δcyo	$\Delta nuo \Delta cyo$
ala	10.314	71.074	11	8.75	8	5.75
arg	5.461	157.174	19.5	15.25	14	9.75
asn	4.177	114.094	11.5	9.75	8.5	6.75
asp	5.923	114.077	11.25	9.5	8.25	6.5
cys	0.817	94.074	9.25	7.25	6.75	4.75
gln	3.952	128.121	16.75	13.5	12.25	9
glu	6.695	128.104	16.75	13.5	12.25	9
gly	8.006	57.047	5.625	4.25	4.125	2.75
his	1.908	137.125	0.25	0.25	0.25	0.25
ile	5.8	113.155	0	0	0	0
leu	8.693	113.155	0	0	0	0
lys	6.403	129.172	0	0	0	0
met	2.65	122.128	0	0	0	0
phe	3.294	147.172	0.25	0.25	0.25	0.25
pro	3.804	97.112	19.25	15.25	13.75	9.75
ser	5.116	87.074	9.25	7.25	6.75	4.75
thr	5.591	101.101	14.625	10.75	10.625	6.75
trp	0.908	186.203	9.5	7.5	7	5
tyr	2.608	163.172	0.25	0.25	0.25	0.25
val	7.878	99.128	0	0	0	0
Weighted average		109.343	7.78	6.18	5.67	4.07
ATP per residue mass (mmol/g)			71.19	56.54	51.84	37.19

Excluding arg, trp:

Weighted average	6.63	5.28	4.84	3.49
ATP per residue mass (mmol/g)	60.66	48.30	44.27	31.90

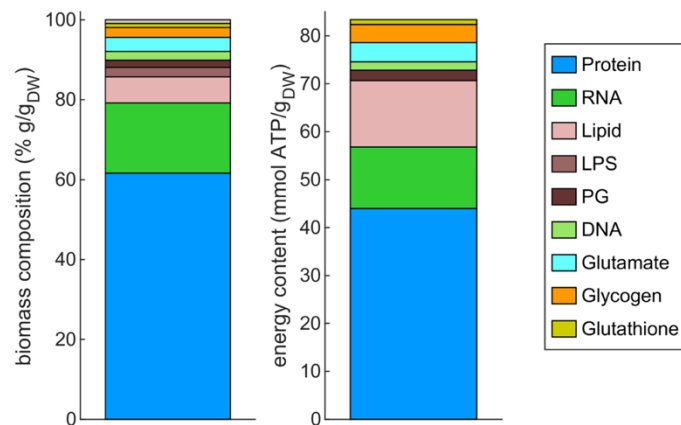


Figure 2.E: Energy content of biomass components

The ATP derivable from each biomass component, listed in Table 2.2: Calculation of energy yield of biomass components, is calculated based on the ATP yield of each monomer using Flux Balance Analysis (FBA), and the biomass composition for *E. coli* cells growing on glucose, taken from⁹². The results are summarized as stacked bars below, with left bar being the biomass composition, and the right bar being the amount of ATP per gCDW.

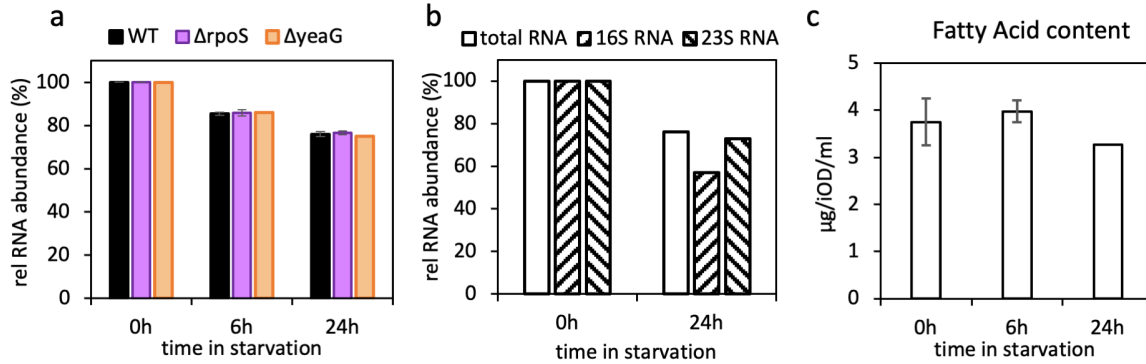


Figure 2.F: Measurements of RNA and lipids

The degradation of RNA in starving bacteria is known for over 60 years⁶⁸ and has remained an active topic of study in bacteriology ever since^{98,105–109}. Most of the RNA degraded is ribosomal RNA (rRNA)^{98,107–109} while tRNAs are relatively stable under various starvation conditions¹¹⁰. The extent to which rRNA is degraded has been estimated by many studies to be between 30-50% within 24h of starvation^{98,107–109,111,112}. There is evidence that several ribosomal proteins require the presence of rRNA for their stability^{96–98}. Molecularly, it is thought that the ClpX-degradation tag that is carried on the C-termini of several ribosomal proteins¹¹³ become exposed in the absence of rRNA, leading to their degradation. However, Kaplan & Apiron¹⁰⁵ showed that ribosomal proteins are fairly stable during starvation, suggesting that either not all r-proteins maybe subject to degradation, or some r-proteins might still be protected by smaller fragments of rRNA. A recent proteomic study corroborated the existence of two r-proteins species, one that degrades at the same rate as rRNA suggesting that they require rRNA for their protection, and the second species that are stable even in the absence of rRNA⁹⁸. The degradation and utilization of lipids by starving bacteria is also an intriguing topic as fatty acid has very high energy content per mass despite its low amount in biomass (Table 2.2). Vast portions of phospholipids were found degraded during starvation by *V. cholerae*¹¹⁴; also interference of fatty acid metabolism is bactericidal and a direction of drug development¹¹⁵. Previous studies in *E. coli* found expression of fatty acid degradation during adaptation to starvation, and mutants unable to make this adaptation exhibited long-term viability loss^{95,116}. Interaction between Acyl carrier protein and SpoT¹¹⁷, the only enzyme that synthesizes and degrades ppGpp in *E. coli*, adds another layer of connection.

For these reasons, the degradation of RNA and lipids were monitored during the course of starvation in addition to proteins. The data in this figure show that they do not play major roles in the conditions we focus on in this study, i.e., during the first 24h of glucose depletion where major proteome remodeling takes place. (a) Total RNA abundance in WT (open black bars), $\Delta rpoS$ (open purple bars), and $\Delta yeaG$ (open orange bars) cultures 6 and 24 hours into glucose starvation, as a percentage of RNA abundance at entry into starvation. Total RNA is measure by the perchlorate method¹¹⁸. RNA abundance in glucose steady state (equivalent to the t=0h, 100% value timepoint here) has previously been established to be ~95 μg/OD/ml⁷⁶. (b) Total RNA is a good indicator of ribosome content during exponential growth as most ribosomes are

expected to be intact. However, during starvation conditions as RNA is degraded, total RNA abundance does not necessarily reflect the intact ribosome content as a few nicks along the rRNA might render the ribosomes without function without causing measurable reduction in total RNA abundance. To gain an understanding on intact ribosome levels, we additionally measured the abundance of full-length 16S and 23S rRNA (striped bars) by TapeStation, an automated electrophoresis method (section Methods 2.6.6). The amounts of total RNA (open bar, from panel a) are shown for comparison. The data indicate that the ribosomes are largely intact for WT cells through the first 24h. (c) Total fatty acid abundance in WT (black bars) cultures that at the time of glucose depletion. The molar abundance of each fatty acid moiety is quantified by gas chromatograph mass spectrometry. The results are weighted by the molecular weight of each species and summed over all species to yield the total fatty acid mass; see Ref. [119] and Methods 2. 6.7. Vertical bars represent standard errors.

2.5 Estimating constraints on protein synthesis during starvation

The maximal amounts of proteins synthesizable from the ATP derivable from measured protein loss are shown in Figure 2.Ae (grey lines), for the maximal conversion efficiency of 8 ATP/AA and one-half of that value. The amounts of protein degradation needed to sustain such synthesis, obtained as the sum of the synthesis amount and the loss amount (circles, Figure 2.Ad), are shown in Figure 2.Af (grey lines). These plots illustrate the substantial cost of protein synthesis for carbon-starved cells, involving the degradation of over half of the pre-starved proteome in the first 24h.

The above energy budget analysis is repeated for $\Delta rpoS$ cells, which showed a similar RNA loss (Figure 2.Fa) but a much-reduced protein loss compared to WT cells (squares, Figure 2.Ad). The data suggests much-reduced ATP regeneration and protein synthesis fluxes (Figure 2.Ae, purple lines), which may be rationalized in two opposing ways: One is that the energy demand is much reduced in $\Delta rpoS$ cells due to their reduced protein synthesis (silencing of the RpoS-regulon). Alternatively, the lack of protein loss by $\Delta rpoS$ cells may reflect their inability to turnover proteins.

2.6 Methods

2.6.1 Growth and Starvation Media

All growth and starvation experiments in this work, including the collection of all proteomics samples, were performed in defined MOPS-buffered minimal medium¹²⁰. The medium includes 40mM MOPS, 4 mM Tricine (adjusted to pH 7.4 by NaOH), 1.32mM KH₂PO₄, 0.1 mM FeSO₄, 0.276 mM Na₂SO₄, 0.5 μM CaCl₂, 0.523 mM MgCl₂, as well as the following concentrations of micronutrients as formulated in (Neidhardt¹²¹): 0.03 μM (NH₄)₆Mo₇O₂₄, 4 μM H₃BO₃, 0.3 μM CoCl₂, 0.1 μM CuSO₄, 0.8 μM MnCl₂, 0.1 μM ZnSO₄·7H₂O. Osmolarity was adjusted using 50mM NaCl¹²². 10mM NH₄Cl was provided as the nitrogen source and glucose was added in limiting or excess amounts depending on the experiment as described in the Cell Growth and Starvation section.

2.6.2 Cell Growth and Starvation

All cell culturing was performed at 37°C in water baths shaking at ~240 r.p.m. To maintain sufficient aeration, 3 ml cultures were grown in 16 mm diameter test tube. For experiments that required larger volumes such as the total RNA assay, proteomics samples, and long-term CFU assays, 10 ml cultures were grown in 20 mm diameter test tubes. Growth rate and viability differences between 3ml and 10ml culture were not distinguishable from noise.

To achieve reproducible, steady-state exponential growth prior to starvation, we followed a standard three-stage culturing approach⁹⁹: seed culture, pre-culture and experimental culture. LB medium was inoculated (seed culture) from a colony on an LB plate

and grown for about 6 hours until there was visible cell growth. Cells were diluted between 10^3 - and 10^4 - fold into MOPS medium (pre-culture) with 10mM glucose such that cultures were in steady-state exponential growth and below an optical density (OD_{600}) of ~ 0.6 after 12-16 hours. The exponentially growing pre-culture was washed in pre-warmed MOPS medium with no glucose and diluted to 0.02 OD_{600} in fresh pre-warmed MOPS medium (experimental culture) with 2 mM or 10 mM glucose depending on whether a run-out or wash-out experiment was being performed.

Starvation via glucose run-out

In glucose run-out experiments, 2 mM glucose was included in the experimental culture such that growth arrested in mid-exponential phase ($OD_{600} \sim 0.4$). Time 0 ($t=0$) of starvation for all run-out experiments was defined as the minute when cultures were measured to exit exponential growth. Due to the rapid exit from exponential growth in glucose cultures at $OD_{600} \approx 0.4$, there is an uncertainty of ~ 5 minutes in the Time 0 value. This uncertainty in time itself is insignificant as our focus is hours after Time 0. However, this uncertainty can introduce up to 10% uncertainty in the OD at Time 0 from one run to another. To avoid this uncertainty affecting the runout OD which all measurements are normalized to, we define the “runout OD” operationally as the OD_{600} reading 15 minutes after the measured Time 0, since by then the increase of OD has surely been arrested. This “runout OD” is also referred to as the “initial OD” or “iOD”.

Starvation via wash

For various assays, washing away the growth media was required to remove components of the growth media no longer wanted during starvation. These assays included: RNA abundance, protein abundance, and protein degradation extent and flux. The goal of a wash-out experiment was typically to replicate a run-out condition. For glucose wash-out experiments, 10mM glucose was included in the experimental culture such that glucose was not limiting during exponential growth. At OD₆₀₀ ~0.4, cells were pelleted via centrifugation (9,000 r.p.m., 3 minutes), washed with 1ml of glucose-free fresh MOPS media, pelleted again, and resuspended in the desired volume of glucose-free fresh MOPS media to achieve an OD₆₀₀ ~0.4 at entry into glucose starvation, similar to run-outs. If washing was needed but a run-out condition desired, for example in certain experiments involving ¹⁴C-leucine, 0.86 mM/0.4 OD₆₀₀ sodium acetate was added in the post-wash medium to mimic the concentration of acetate due to overflow metabolism.

2.6.3 Strain construction

Bacterial strains used in this study are described in Table 2.4. The wildtype strain is NCM3722^{85,123}; all other strains were derived from this parental strain. Single structural genes *dps*, *yeaG*, *aceA*, *yeaH*, *clpP* and *leuA* were individually deleted from NCM3722 by P1 transduction using appropriate kanamycin (Km) resistant mutants (JW0797-1, JW1772-1, JW3975-3, JW1773-1, JW0427-1 and JW0073-1) from Keio Collection as donors. In some cases, the Km resistance gene (*km^r*) was flipped out from P1 transductants by pCP20¹²⁴. This yielded six deletion mutant strains HE961, HE845, NQ1026, HE988, HE1017 and HE1057, in which *dps*, *yeaG*, *aceA*, *yeaH*, *clpP* and *leuA* were deleted, respectively (Table 2.4).

Each double mutant was made by P1 transducing one mutation to a recipient strain harboring the other mutation. Using this strategy, the following double mutants were yielded: (1) HE963 (deleted for *yeaG* and *aceA* by transferring the *aceA* mutation into the *yeaG* single mutant); (2) HE969 (deleted for *dps* and *aceA* by transferring the *aceA* mutation into the *dps* single mutant); (3) HE968 (deleted for *rpoS* and *aceA* by transferring the *aceA* mutation into the *rpoS* single mutant); and (4) HE962 (deleted for *dps* and *yeaG* by transferring the *yeaG* mutation into the *dps* single mutant).

For the *lon/hslV* mutant, A strain annotated as *lon/clpP/hslV* triple mutant was obtained from the Markus Basan Lab. PCR and sequencing analyses revealed that this mutant carries the complete *clpP* gene. Therefore, this so-called triple mutant was actually a *lon/hslV* double mutant, which is now named HE996 (deleted for *lon* and *hslV*).

For the *clpP/clpX/lon* mutant, the contiguous chromosomal region carrying *clpP*, *clpX* and *lon* was deleted using the Lambada-Red method¹²⁴. The *kmr* gene was amplified from pKD4 using chimeric oligos ClpP-P1 and Lon-P2 (Table 2.5).

The purified PCR products were electroporated into NCM3722 cells expressing Lambada-Red proteins encoded by pKD46. The Km resistant colonies were confirmed by PCR for the replacement of genes *clpP*, *clpX* and *lon* by *km^r*. This yielded strain HE1014, in which *clpP*, *clpX* and *lon* are deleted.

The triple ribosome hibernation mutant strain HE587 was made by two rounds of P1 transduction, first by combining *rmf* and *hpf* mutations in NCM3722 followed by transferring the *raiA* mutation into the *rmf/hpf* double mutant.

To create the mutations of *yeaG*_E263A (the 81st codon GAG changed to GCG) or *yeaG*_K426A (the 132nd codon AAG changed to GCT) on chromosome, two major steps were employed. First, the *yeaG* gene plus its promoter region were deleted. Second, the same DNA fragment harboring the E263A or K426A mutation was moved back to the chromosome at the same *yeaG* locus.

Using the same Lambda-red approach, a 1656-bp chromosomal region, located at -351 to +1305 with respect to the *yeaG* translation start site, was first replaced by a *km^r* gene that was then flipped out by pCP20. This yielded strain Δ *pyeaG-yeaG*.

Fusion PCR was used to introduce the E263A alteration to the *yeaG* gene carried in the *pyeaG-yeaG* fragment. First, two fragments sharing a 43-bp overlapped region in between, *pyeaG-yeaG'*_E263A (harboring the E263A mutation at the 3' end) and E263A_'*yeaG* (harboring the E263A mutation at the 5' end), were individually amplified from the NCM3722 genomic DNA. These two fragments were then fused together by PCR. The fused product, *pyeaG-yeaG*_E263A (that is, the entire *pyeaG-yeaG* fragment carrying the E263A mutation in *yeaG*) was ligated with a DNA fragment (carrying a *neo* gene and the *sc101 ori*) amplified from the low-copy plasmid pZS24¹²⁵, yielding pZS24_*pyeaG-yeaG*_E263A. Using this plasmid as the template, the *pyeaG-yeaG*_E263A fragment plus the upstream *neo* gene (that is, *neo::pyeaG-yeaG*_E263A) was amplified. The purified products were introduced into strain Δ *pyeaG-yeaG* that over-expressed the Lambda-red proteins. Several Km resistant colonies were verified first by PCR and then by sequencing for the integration

of *pyeaG-yeaG_E263A* at the *yeaG* locus. This yielded strain HE1006, in which the *yeaG* carries the desired E263A mutation at its native locus.

Similarly, the “*neo::pyeaG-yeaG_K426A*” cassette was constructed and then integrated onto the chromosome, yielding strain HE1008, in which the *yeaG* carries the K426A mutation. In both strains HE1006 (harboring *yeaG_E263A* point mutation) and HE1008 (harboring *yeaG_K426A* point mutation), the modified *yeaG* gene (with only one codon alteration) is located at its native locus and it has no other mutations except a *neo* gene (encoding Km resistance) situated immediately upstream of -351.

To make strain NQ537, the cassette “*km^r:Pu-lacZ*” (i. e., the σ^{54} promoter Pu driving *lacZ* at the *lac* locus) was P1 transduced from strain EQ48 ($\Delta lacI \Delta lacY$)¹²⁶ to strain NQ386¹²⁷ which carries a Crp-independent promoter *P_{lac}* driving *xylR* at the *attB* locus. In the resultant strain NQ537, the *lacZ* transcription is positively regulated by the constitutively expressed XylR, whose activity is dependent on the levels of 3MBA (3-methylbenzylalcohol, the inducer for XylR).

To make strain HE1010, the deletion of *lacI/lacZ/lacY* (that is, a *km^r* gene replacing these three genes) was first transferred from strain EQ356¹²⁸ to strain NQ386. The resultant strain was transformed with the *lacZ* overexpression plasmid pZE12cam/*Pu-lacZ*⁷. In the final strain HE1010, XylR (after being activated by 3MBA) dictates *lacZ* expression by activating the Pu promoter.

2.6.4 Viability assay

Viability was measured by counting colony forming units (CFU) on Luria Broth (LB) agar plates. Details of plate preparation were important for reproducible results.

Preparation of LB agar plates for viability curves

80g LB agar powder was mixed with ddH₂O to 2 liters. The solution, along with a magnetic stir bar, was autoclaved for 30 minutes at 120°C. Upon the autoclave cooling to ~80°, the LB agar solution was moved onto a stir-plate at room temperature and stirred until the temperature of the media cooled to ~60°C, before agar began to solidify. Near a flame for sterility, 30ml of the LB agar solution was dispensed into sterile 100x15mm petri dishes using a manual 25ml pipette such that no bubbles were present on the plate surface. Plates were left face up, partially covered by a lid until the solution solidified. Lids were placed on the plates, and they were flipped upside down (agar side up, to avoid excess water loss) to dry for ~20 hours at room temperature in stacks of 5 plates each. Any condensation on plate lids was wiped off with a Kimwipe and plates were stored upside down (agar side up) in stacks of 20-25 at 4° in plastic plate sleeves for up to 1 week.

Preparation of plates for cell plating

Plates were removed from storage at 4°C ~40 minutes-1hr before cell plating and warmed in a 37°C incubator upside down (agar side up) for 25 minutes. They were then dried face up with lids removed for 5 minutes in a PCR hood. Plates were then transferred with lids to a 37° incubator and stored upside down (agar side up) for ~a few minutes-1 hour.

Preparation of cells for plating

Cultures at various time into starvation were diluted in glucose-free MOPS media to ~100 CFU/100 μ l. Cells were diluted via 10X serial dilution, sequentially mixing 100 μ l of sample in 900 μ l of MOPS media with 10mM NH₄Cl and no glucose. To yield a final concentration of 100 CFU/100 μ l, the final dilution step varied depending on runout OD₆₀₀ and the viability of the cell population at that timepoint based on previous measurements. For each sample, 100 μ l of diluted culture was pipetted on to three LB plates at each timepoint to obtain technical replicates. Diluted culture was spread on the plate with 5 glass beads. After spreading, diluted culture absorbed into the plate within minutes. Plates were incubated upside down (agar side up) for 20-24 hours in a 37° incubator until visible colonies formed.

Analysis of culture viability

Colonies (CFU) were counted manually. Each biological timepoint plotted throughout the manuscript represents the average of three technical replicates. Biological replicates were performed in separate tubes on different days. Biological replicates are binned into ~2-hour windows. Unless otherwise noted, all data is normalized to CFU at 6 hours into glucose starvation to account for reductive division during the first few hours of runout.

2.6.5 Relative protein abundance

The scheme for measuring relative protein abundance is described in Appendix A1. 3 ml cultures were grown in the presence of limiting glucose (2 mM) and 50 μ M ¹⁴C-leucine

[at 6.7 μ Ci/ μ mol]. The addition of leucine was confirmed to not affect growth, yield, and survival of *E. coli* (Appendix A2) Furthermore, leucine uptake was comparable between wild type and a leucine auxotroph (HE1057) suggesting that the externally supplied leucine is the only source of leucine incorporated into proteins. Upon glucose runout, cells were washed to remove all external leucine and resuspended in MOPS buffer without glucose and leucine but supplemented with 0.86mM acetate to make up for the acetate released during growth, unless specified otherwise. At various time intervals 100 μ l of the culture was withdrawn and precipitated with 200 μ l of 20% Trichloroacetic acid (TCA) for at least 30 minutes on ice. One half of the precipitation reaction was then centrifuged at maximum speed to separate the precipitate and the supernatant. The supernatant and the other half of the reaction were mixed with 2ml scintillation cocktail (Liquiscint™, National Diagnostics), and their radioactivity were measured using a Beckman LS6500 counter. The difference between the radioactivity in the total reaction and the supernatant was taken to be the radioactivity of the protein precipitate. This method of subtractive estimation allowed us to bypass the typical need to repeatedly wash the precipitate with acetone¹²⁹ which in our hands lead to 30-60% loss of material. The precipitate radioactivity at various times relative to that at the time of glucose runout was taken as the relative protein abundance. We note that we were unable to reliably recover TCA precipitable material below culture OD₆₀₀ of 0.1 (Appendix A2b) and thus ensured that all experiments were carried out at OD₆₀₀ 0.2-0.4.

2.6.6 Total RNA, 16S and 23S rRNA abundance

Total RNA and 16S and 23S rRNA abundance in cultures were measured during growth and at various time points during starvation. Total RNA mass in cultures was measured using the perchlorate method¹¹⁸, with minor modifications as described in Ref.[127], yielding total RNA $\mu\text{g/ml}$ culture. Briefly, 10 ml samples were collected by centrifugation (21,000 r.p.m. for 3 minutes), cell pellets washed twice with cold 0.7M HClO_4 and digested with 0.3 M KOH for 1 hour at 37°. Cell digestion was halted by addition of cold 3M HClO_4 , samples centrifuged (21,000 r.p.m. for 3 minutes) and supernatant collected, including supernatant from two pellet washes with cold 0.5M HClO_4 . Supernatant OD_{260} was measured using a spectrophotometer. For 16S and 23S rRNA, total RNA was extracted at the desired timepoints using the TRIZOL reagent (ThermoFisher, catalog number 15596018) according to the vendor's protocol and the samples were evaluated using Agilent TapeStation at the UCSD Institute of Genomic Medicine (IGM) core facility.

In both RNA assays, $t=0$ samples were collected during exponential growth between 0.3 and 0.4 OD_{600} in order to capture RNA abundance before entering starvation. RNA abundance was normalized to the $t=0$ OD_{600} . For time points during starvation, RNA abundance was normalized to the OD at growth arrest (iOD defined above), yielding RNA abundance/iOD/ml. RNA abundances/(i)OD/ml are presented as a percentage of RNA abundance/OD/ml at $t=0$.

2.6.7 Fatty acid abundance

10ml cultures were collected during growth and at various time points during starvation via centrifugation (21,000 r.p.m., 3 minutes). For the t=0h time point, samples were collected during exponential growth between 0.3 and 0.4 OD₆₀₀ before entering starvation. Fatty acid analysis was performed at the UCSD Lipidomics Core. Total fatty acid is obtained by adding up the abundances of all fatty acid $\mu\text{g}/\text{OD}\cdot\text{ml}$ obtained from GC-MS analysis following the method described in¹¹⁹.

2.6.8 Methods Tables

Table 2.4: *E. coli* strains used in this work

All strains used throughout this work. Single, double and triple knockouts were cloned into our WT strain (NCM3722) using P1 transduction with the Kanamycin knockout cassette originating from the BW25113 Keio collection¹³⁰ (Section 2.6.3). Novel gene mutations were performed using the recombineering method¹²⁴ using the primers listed in Table 2.5.

Strain name	Strain Description	Reference
NCM3722	Wild type	(85,123)
NQ1191	$\Delta rpoS::km$	(78)
HE961	Δdps	This work
HE587	$\Delta rmf, \Delta hpf, \Delta raiA$	This work
HE845	$\Delta yeaG::km$	This work
NQ1026	$\Delta aceA$	This work
HE963	$\Delta yeaG, \Delta aceA$	This work
HE969	$\Delta dps, \Delta aceA$	This work
HE968	$\Delta rpoS, \Delta aceA$	This work
HE988	$\Delta yeaH$	This work
HE1017	$\Delta clpP$	This work
HE1014	$\Delta clpXP, \Delta lon$	This work
HE996	$\Delta lon, \Delta hslV$	This work
HE1006	$pyeaG_yeaG$ (E263A)	This work
HE1008	$pyeaG_yeaG$ (K426A)	This work
HE962	$\Delta dps::km, \Delta yeaG$	This work
HE1057	$\Delta leuA-$	This work
NQ537	$P_{llac-xylR}, Pu-LacZ, \Delta lacIY$	(8)
HE1010	$P_{llac-xylR}, \Delta lacIZY, pZE12-Pu-lacZ$	This work

Table 2.5: Oligos used in this work

Name	Sequence	Use
ClpP-P1	acgcgtcattttctgactggccag gttgaagaccacatggctaacctg attgtgtaggctggagctgcttc	Deletion of <i>clpP/clpX/lon</i>
Lon-P2	cagagtcagaacttcctcaatgcg cttcacaggatgaatgtccagatc ggcaattccggggatccgtcgac ctg	Deletion of <i>clpP/clpX/lon</i>
Lon-ver-R	acttgaatccttcaaggtagcaac g	Verification of deletion of <i>clpP/clpX/lon</i>
PyeaG-P1	cgacaaagtttaccgctcaatac atcgaaacccaacctttttacgtt tcatgtgtaggctggagctgcttc	Deletion of <i>pyeaG-yeaG</i>
YeaG-P2	ctgttcaggacgtagaacagatg gaccgggtttgctgccacttctac atgattccggggatccgtcgacct g	Deletion of <i>pyeaG-yeaG</i>
YeaF-ver-R	agacgttgcgataagcactccaa g	Verification of deletion of <i>pyeaG-yeaG</i>
YeaG-ver-R	ttaagacgatttacgtacgcgcaa atacc	Verification of deletion of <i>pyeaG-yeaG</i>
PyeaG-Xho-F	atactcgagtaactgtttaatttcc tgacgcggatgac	Amplification of <i>pyeaG-yeaG</i> , <i>pyeaG-yeaG'</i> _E263A or <i>pyeaG-yeaG'</i> _K426A
YeaG-E263A-R	cactttaatcgggtgctttaaacatc gcaacgaattccatgatcc	Amplification of <i>pyeaG-yeaG'</i> _E263A
YeaG-E263A-F	gatcatggaattcgttgcgatgttta aagcaccgattaaagtg	Amplification of E263A_' <i>yeaG</i>
YeaG-Bam-R	aatggatccttaagacgatttacgt acgcgcaaatacc	Amplification of <i>pyeaG-yeaG</i> , E263A_' <i>yeaG</i> or K426A_' <i>yeaG</i>
YeaG-K426A-R	gttgaacacgcgggagaggatag caaacgcaaacgcgtcgacag ac	Amplification of <i>pyeaG-yeaG'</i> _K426A
YeaG-K426A-F	gacgcgttttgcgtttgctatcctct cccgcgtgttcaac	Amplification of K426A_' <i>yeaG</i>
pZS-Bam-F	aatggatcccattggtacgcgtgct agaggcatc	Amplification of pZS24
pSS-Xho-R	atactcgagattccacacagacg tcggaattgccagctg	Amplification of pZS24

Table 2.5: Oligos used in this work

Name	Sequence	Use
Pyg-neo-P1	ggcgacaaagtttaccgtcaata catcgaaacccaaccttttacgtt ttcagaagaactcgtcaagaagg cgatag	Chromosomal integration of pyeaG- yeaG_E263A or pyeaG-yeaG_K426A
YeaG-P3	ctcctgatacctgaatccagaaatc cgcgtag	Chromosomal integration of pyeaG- yeaG_E263A or pyeaG-yeaG_K426A
YeaG-ver-R3	ttgcggaaatcttttggtactgat c	Verification of pyeaG-yeaG_E263A or pyeaG-yeaG_K426A
YeaG-ver-F1	aaggtctggccgtcaattctgcaa c	Verification of pyeaG-yeaG_E263A or pyeaG-yeaG_K426A
YeaG-ver-F2	atgaagggatgaacggtctgtcga cg	Verification of pyeaG-yeaG_E263A or pyeaG-yeaG_K426A
YeaG-ver-R1	agcgcaccggaatagccgtaggc gtc	Verification of pyeaG-yeaG_E263A or pyeaG-yeaG_K426A
YeaG-ver-R2	tcagataagagacaatctgttcaa tcg	Verification of pyeaG-yeaG_E263A or pyeaG-yeaG_K426A

2.7 Acknowledgements

With permission from co-authors, this chapter reproduces materials and analysis, in part, from the following paper in preparation: Manzanarez, Gabriel[†]; Balakrishnan, Rohan[†]; Kannan, Harish[†]; Abele, Miriam; Zhang, Zhongge; Wu, Chenhao; Freiman, Lance; Enuston, Eray; Corbett, Kevin D.; Ludwig, Christina ^{*}; Mori, Matteo ^{*}; Hwa, Terence^{*}. “YeaG-mediated global proteolysis in starving bacteria dictates protein synthesis and prolongs cell survival”. The dissertation author was the primary author of this paper.

Chapter 3: Proteome remodeling by WT and $\Delta rpoS$ cells

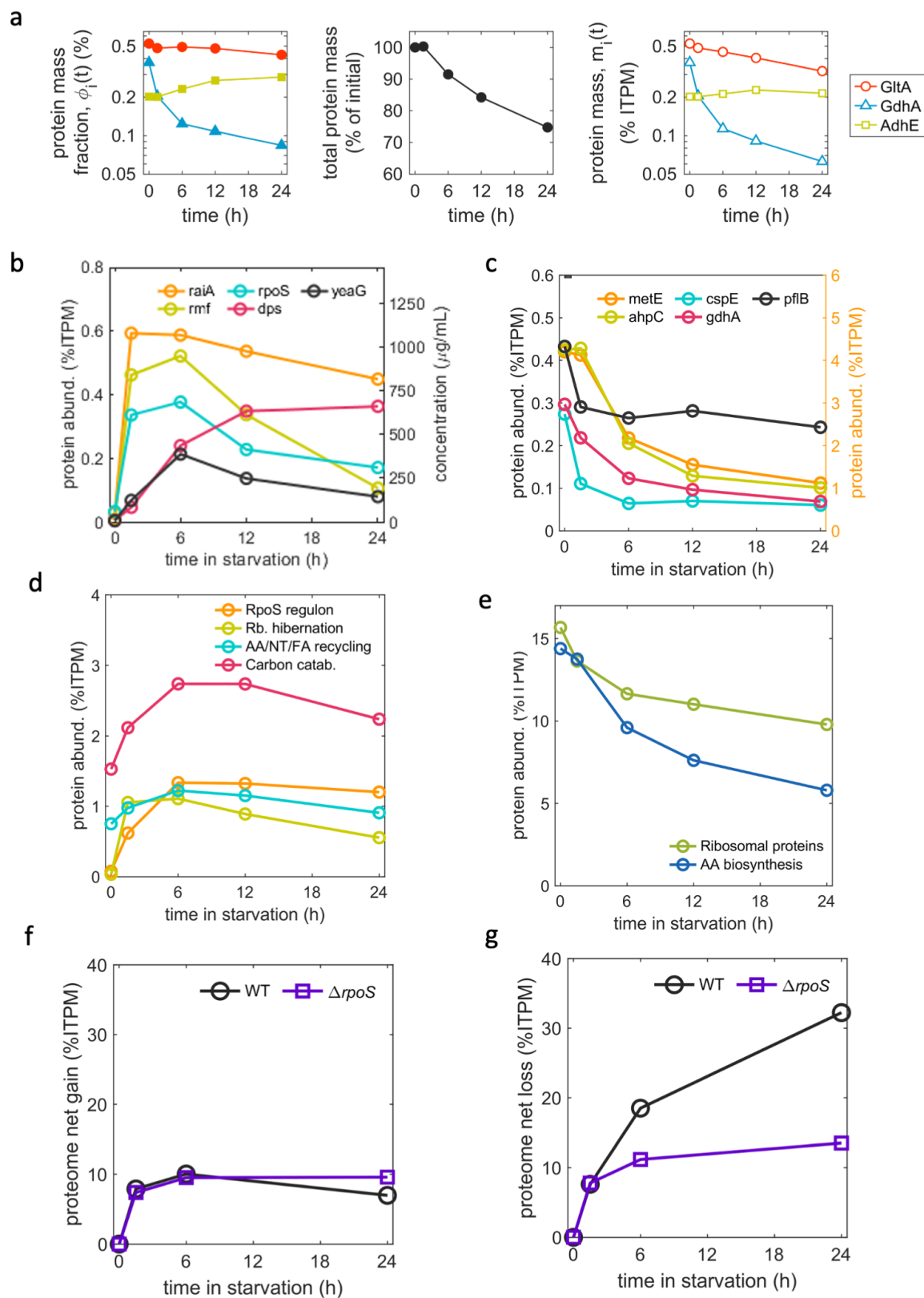
To understand the substantial difference in protein turnover between WT and $\Delta rpoS$ cells and its implication on proteome remodeling, we performed proteomic analysis for these and other strains after glucose depletion using data-independent acquisition (DIA) mass spectrometry^{131,132}. The proteomic data collected, expressed in terms of the mass fraction of individual proteins⁸⁶ (Table 2.1) are quantitative and reproducible. Combining this data with the measurements of total protein abundances (Figure 3.A), we obtained the time courses of the absolute abundances of over 2000 proteins during the first 24h of starvation.

3.1 Analysis of WT proteome during first 24 hours of starvation

First, we provide an analysis of the WT cells: Despite the decrease of total protein amount in the WT culture (Figure 2.Ad), ~150 proteins exhibited increases > 0.01% ITPM (~400 copies per cell, see Table 2.1) by 6h after starvation (red symbols, Figure 3.B). Among the proteins with the largest increase at that time are RpoS and several members of the RpoS-regulon (Dps^{133,134}, YeaG), the ribosome hibernation factors (Rmf, RaiA)¹³⁵ and several enzymes involved in carbon catabolism. The time courses of the five proteins that increased the most amount are shown in Figure 3.Ab. We also found ~300 proteins which decreased > 0.01 %ITPM by 6h after starvation (blue symbols, Figure 3.Ba). These proteins are dominated by metabolic enzymes and components of the translational apparatus, with the time-courses of the five proteins that decreased the most amount shown in Figure 3.Ac, including MetE, the most abundantly expressed protein during exponential growth on glucose^{136,137}.

Figure 3.A: Proteome remodeling by WT and $\Delta rpoS$ cells during glucose starvation

(a) Illustration of absolute abundance estimation for individual proteins. From protein samples collected at time $t=0$ (initial), 1.5h, 6h and 24 h during glucose starvation, mass-spectrometry based proteomics⁹⁹ gives the fractional abundance of each detected protein, $\phi_i(t) = m_i(t)/m_{total}(t)$, where $m_i(t)$ is the mass of an individual protein i , and $m_{total}(t) = \sum_i m_i(t)$ is the total protein mass detected. Because proteins captured by proteomics account for over 90% of total protein mass in the culture⁸⁶, we take $m_{total}(t)$ to be the biochemically measured protein mass $M(t)$ as given in Figure 2.Ad, and estimate $m_i(t)$ as $\phi_i(t) \cdot M(t)$. The result is plotted as %ITPM (defined in Figure) for three exemplary proteins. (b, c) The time course of protein mass $m_i(t)$ in WT culture for five proteins with largest increase or decrease between the onset of glucose starvation and 6h into starvation, excluding proteins with <50% change. The right axis in panel (b) indicate the equivalent concentration for an average protein 250aa in size⁸⁷; the right axis in panel (c) indicates the abundance of MetE. (d) The time course of summed mass of proteins belonging to the four functional groups (that increased in WT culture during the first 6h of glucose starvation. (e) Same as (d), but for two groups that show by far the largest decreases. (f) The time course of the sum of all detected proteins whose mass increased from that at the time of starvation. Circles: WT culture; squares: $\Delta rpoS$ culture. (g) Same as (f), but for proteins whose mass decreased. For (b-g) the left y-axis represents protein mass in % ITPM and the right y-axis represents the same protein mass in ug/mL with runout OD being 0.4. The error bars on individual protein masses denote the standard deviation which reflect the scatter in the peptide precursors detected by mass-spectrometry and a basal noise level originating from the uncertainty in the absolute scale of protein abundance⁹⁹. The error bars on *group mass* are estimated by treating individual protein masses within the group as independent random variables $\sigma^2 = \sum_i$.



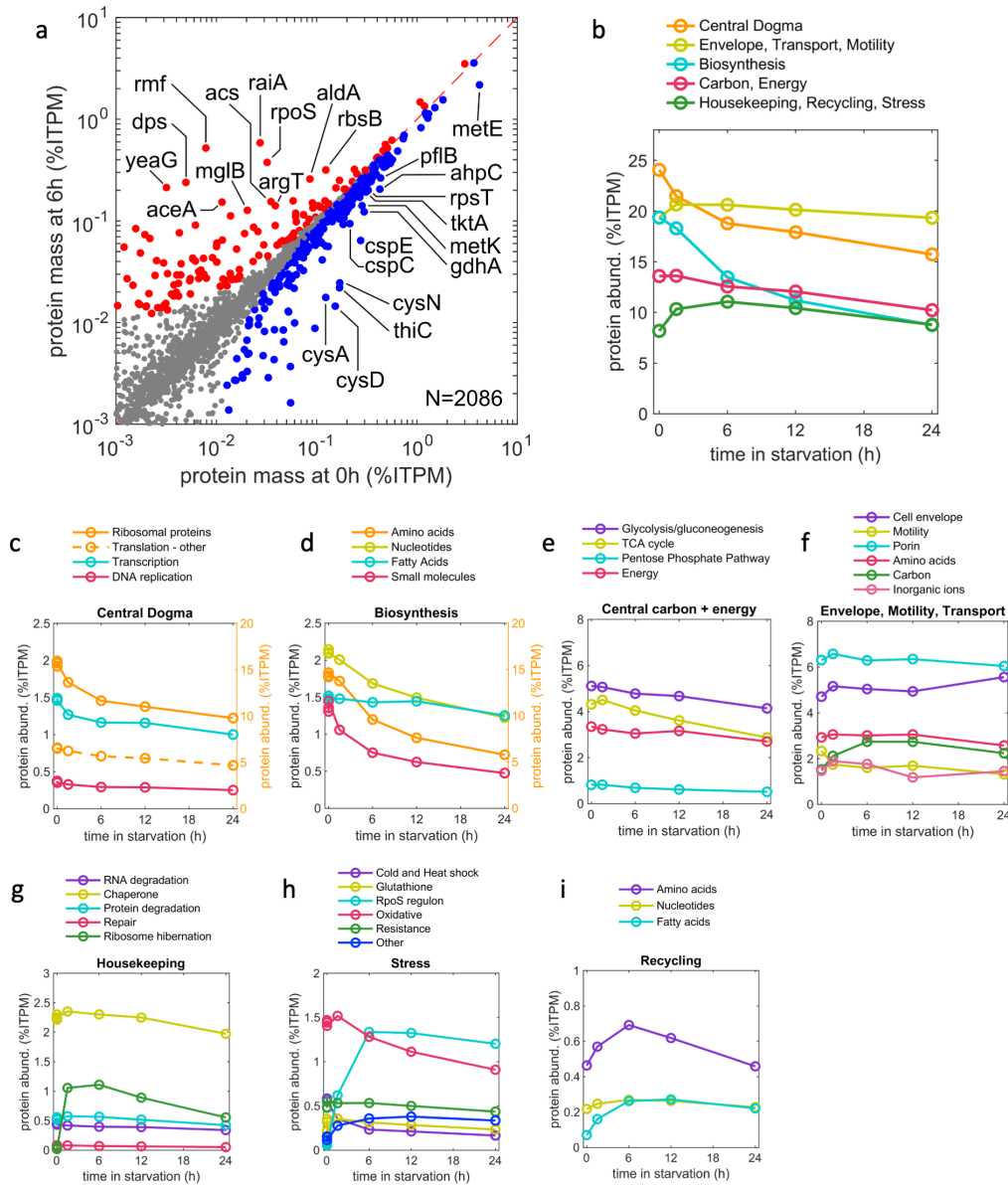


Figure 3.B: Proteome remodeling by WT cells during starvation.

(a) Change in protein levels, expressed as percent of the total initial protein mass (% ITPM), for wild type cells between the onset of starvation (at 0h) and 6 hours after glucose runout. Proteins which increased (N=157) or decreased (N=298) by more than 0.01% ITPM were highlighted in red and blue, respectively; protein names are shown for proteins with changes above 0.1% ITPM. Note: 0.01% ITPM corresponds to about 400 copies/cell; see Table 2.1. (b) The total abundance of proteins belonging to the five coarse-grained categories over time after entry into starvation. (c-i) Breakdown of each of the five categories shown in panel (b) into the corresponding subgroups. The “housekeeping, recycling, stress” category in panel (b) is broken down in more detail, into three separate panels (g-i), each into its subgroups.

A simplified view of the protein dynamics can be gained by following the total abundances of various protein functional groups. We classified proteins into 5 categories, each further divided into 5-6 groups; altogether this classification accounts for ~1200 proteins that comprise ~85% of the proteome in steady state growth. The time course of the total protein abundance of each category is shown in Figure 3.Bb, and the abundance of each group in Figure 3.Bc-i, with exemplary group members shown in Figure 3.C. Figure 3.Ad shows the abundances of groups that gained abundances in the first 6h: the RpoS-regulon, ribosome hibernation factors, and carbon catabolism each gained 1-1.5% ITPM, while recycling enzymes gained 0.5% ITPM. Groups that decreased were dominated by amino acid biosynthesis (AAB) enzymes and ribosomal proteins (Figure 3.Ae). The net gain and loss by all detected proteins are shown as circles in Figure 3.Af, Figure 3.Ag, respectively, revealing that a lot more proteins have reduced abundances than increased abundances, consistent with the general expectation based on resource allocation (Figure 2.Ae, Figure 2.Af). However, it should be noted that net gain/loss provide only lower bounds for synthesis/degradation, since a protein could be synthesized and degraded as will be elaborated further below.

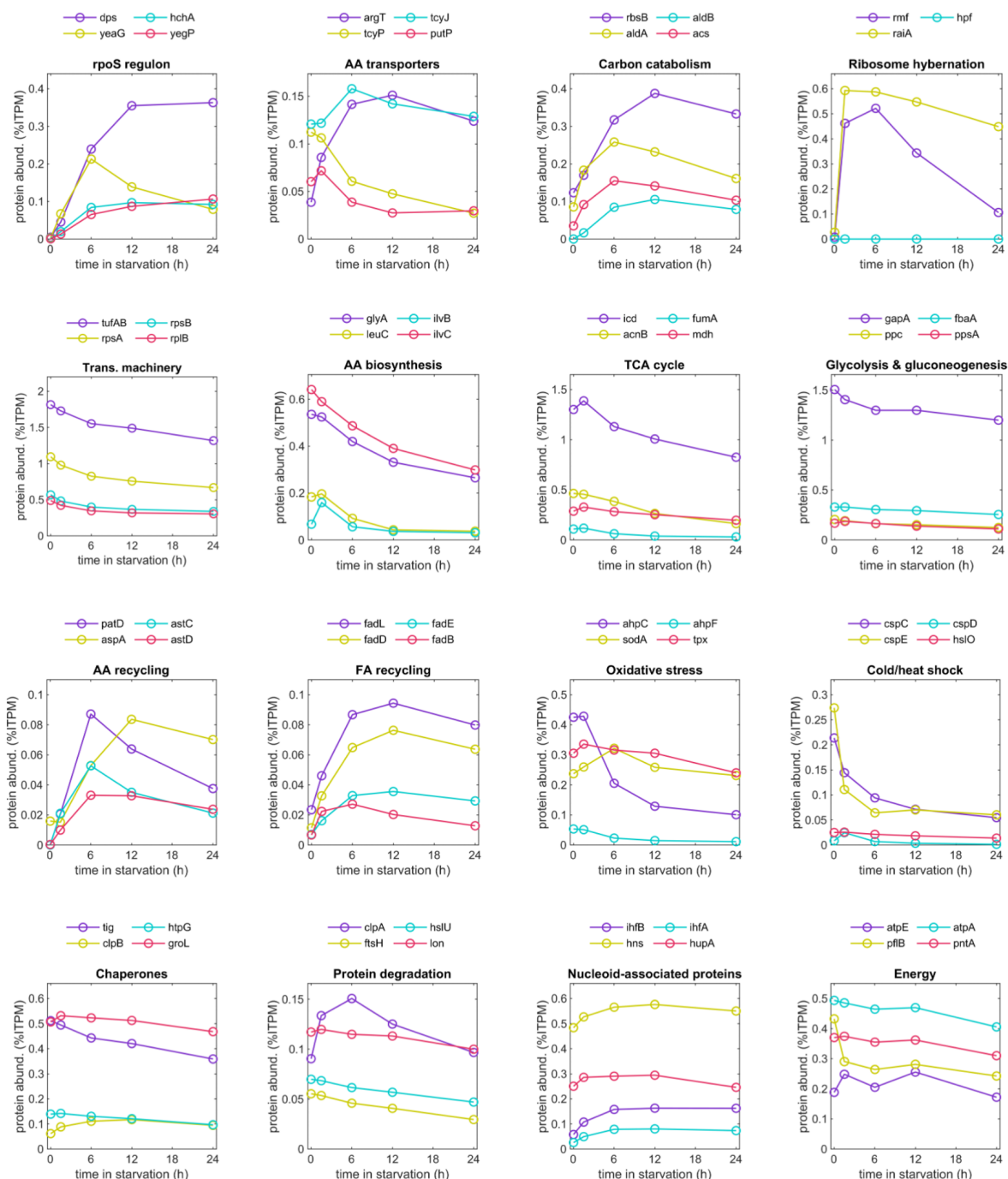


Figure 3.C: Changes of exemplary proteins in each functional group, WT cultures

For each of 16 selected protein groups among those shown in Figure 3.Bc-i, the time courses of the top 4 proteins with the largest change in amount between 1.5 and 6 hours of starvation are plotted. In the translation machinery subgroup, tufAB represents the overall abundance of the elongation factor EF-Tu.

3.2 Analysis of $\Delta rpoS$ proteome during first 24 hours of starvation

We repeated the proteome analysis for $\Delta rpoS$ cells. The net loss by $\Delta rpoS$ cells is much smaller than that of WT cells (Figure 3.Ag). An analysis of the proteome (Figure 3.Da-e) reveals that the loss in ribosomal proteins and biosynthetic enzymes is suppressed in $\Delta rpoS$ cells after the first several hours (Figure 3.Da, b), and this difference accounts for most of the net protein loss. On the other hand, the net protein gain by $\Delta rpoS$ cells is surprisingly similar to that of WT (Figure 3.Af). Analyzing the composition of the synthesized proteome reveals that the two strains differ in which proteins are synthesized (Figure 3.Df-g), with $\Delta rpoS$ cells losing recycling enzymes in addition to the RpoS regulon, but gaining in biosynthetic and TCA cycle enzymes which are not needed during starvation (possibly reflecting the lack of sigma factor competition in $\Delta rpoS$ cells). Regardless, the similar amounts of proteins gained by $\Delta rpoS$ and WT cells^{138,139} demonstrates that there is active protein synthesis in $\Delta rpoS$ cells. Thus, the reduced total protein loss observed in $\Delta rpoS$ cells (Figure 2.Ad) cannot be explained by a shutdown of protein synthesis and concomitant shutdown of degradation. Rather, protein degradation seems crippled in $\Delta rpoS$ cells after several hours of starvation.

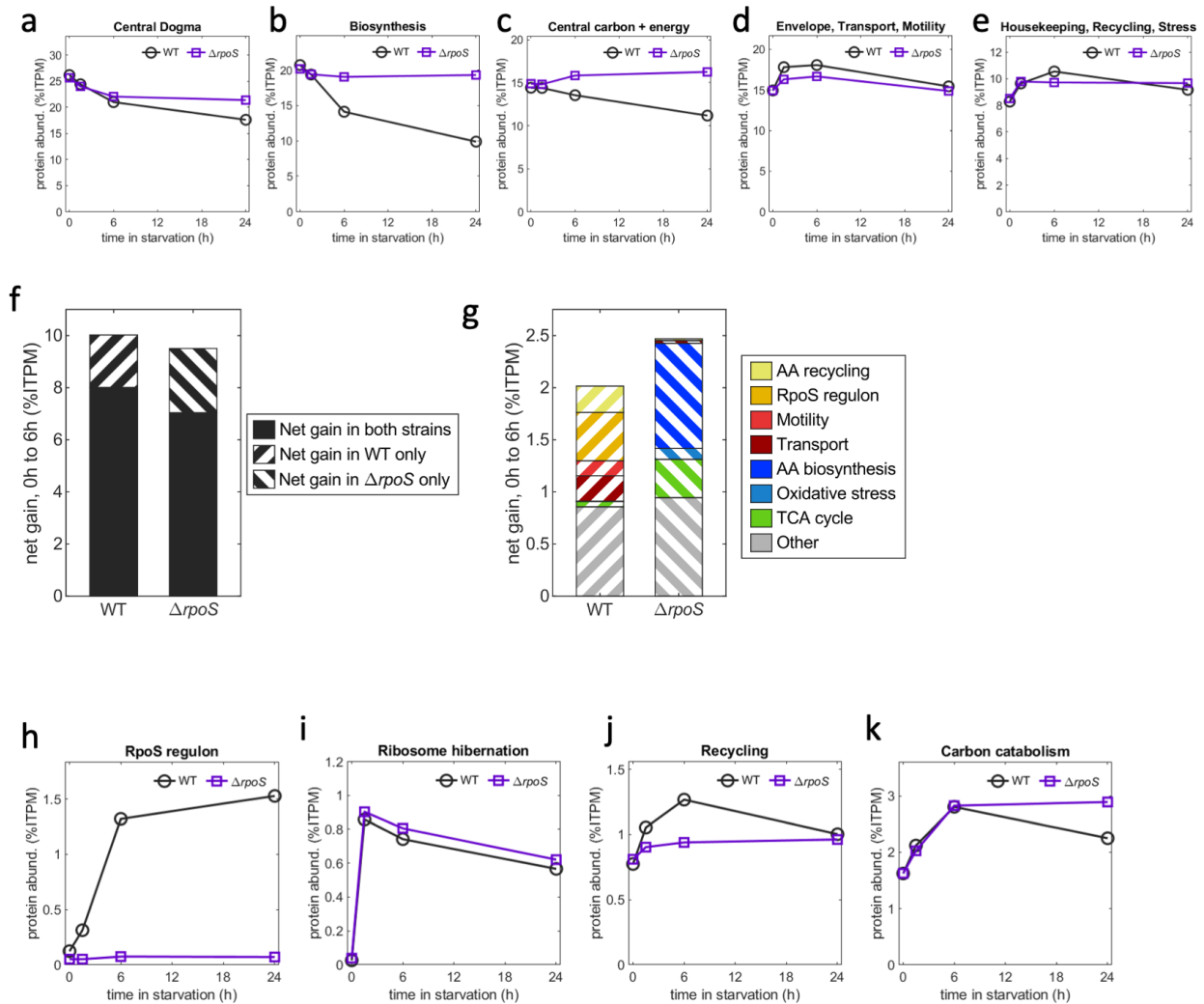


Figure 3.D: Proteome remodeling by $\Delta rpoS$ cells

(a-e) Remodeling of the five main functional categories for wild type and $\Delta rpoS$ cells during glucose starvation. Proteomics data was collected and processed in the same batch (see methods). (f) The net protein gain at 6h of starvation, obtained by summing over the abundances of all proteins which gained in abundance compared to exponential growth (onset of starvation) was similar for WT and $\Delta rpoS$ cells. Black bars indicate the total gain originating from proteins with net gain in both strains; striped bars indicate contributions from proteins with net gain in one strain but not in the other. (g) Breakdown of net protein gains unique to either strain (the striped bars in the previous panel) into individual protein groups. (h-k) Changes in the total abundances of the groups which gained in WT cells (the groups shown in Figure 3.Ab).

3.3 Methods

3.3.1 Preparation of protein samples for mass spectrometry

For proteomic analysis, cultures were grown using the standard MOPS media and cell growth protocol described above and samples were prepared using a modified version of the method described in Ref. [140]. 10 ml of culture were harvested via centrifugation (21,000 r.p.m., 3 minutes) at various time-points during glucose starvation such that visible pellet was observed. T=0, steady-state, samples were collected at OD₆₀₀ ~0.4 and cultures entered starvation at OD₆₀₀ ~0.4. Cell pellets were washed once with 150 mM NaCl, rapidly frozen on dry ice and stored at -80° C. Cell lysis was performed by adding 75µl of 100% TFA to each cell pellet in a laminar flow fume hood, pipette mixing, and heating pellets on a heat block at 55°C for precisely 5 minutes. After 5 minutes, 2M pH-unadjusted Tris-base was added with brief vortex mixing to neutralize the dissolved cell pellet in a laminar flow fume hood. Lysed samples were stored at -80C.

3.4 Acknowledgements

With permission from co-authors, this chapter reproduces materials and analysis, in part, from the following paper in preparation: Manzanarez, Gabriel[†]; Balakrishnan, Rohan[†]; Kannan, Harish[†]; Abele, Miriam; Zhang, Zhongge; Wu, Chenhao; Freiman, Lance; Enuston, Eray; Corbett, Kevin D.; Ludwig, Christina ^{*}; Mori, Matteo ^{*}; Hwa, Terence^{*}. “YeaG-mediated global proteolysis in starving bacteria dictates protein synthesis and prolongs cell survival”. The dissertation author was the primary author of this paper.

Chapter 4: YeaG enables an early wave of protein degradation

The arrest in the loss of many proteins in $\Delta rpoS$ cells suggest the possibility that a member of the RpoS-regulon mediates the extensive protein loss found in WT cells during starvation (Figure 2.Ad).

4.1 Identifying the driver of increased protein degradation in WT

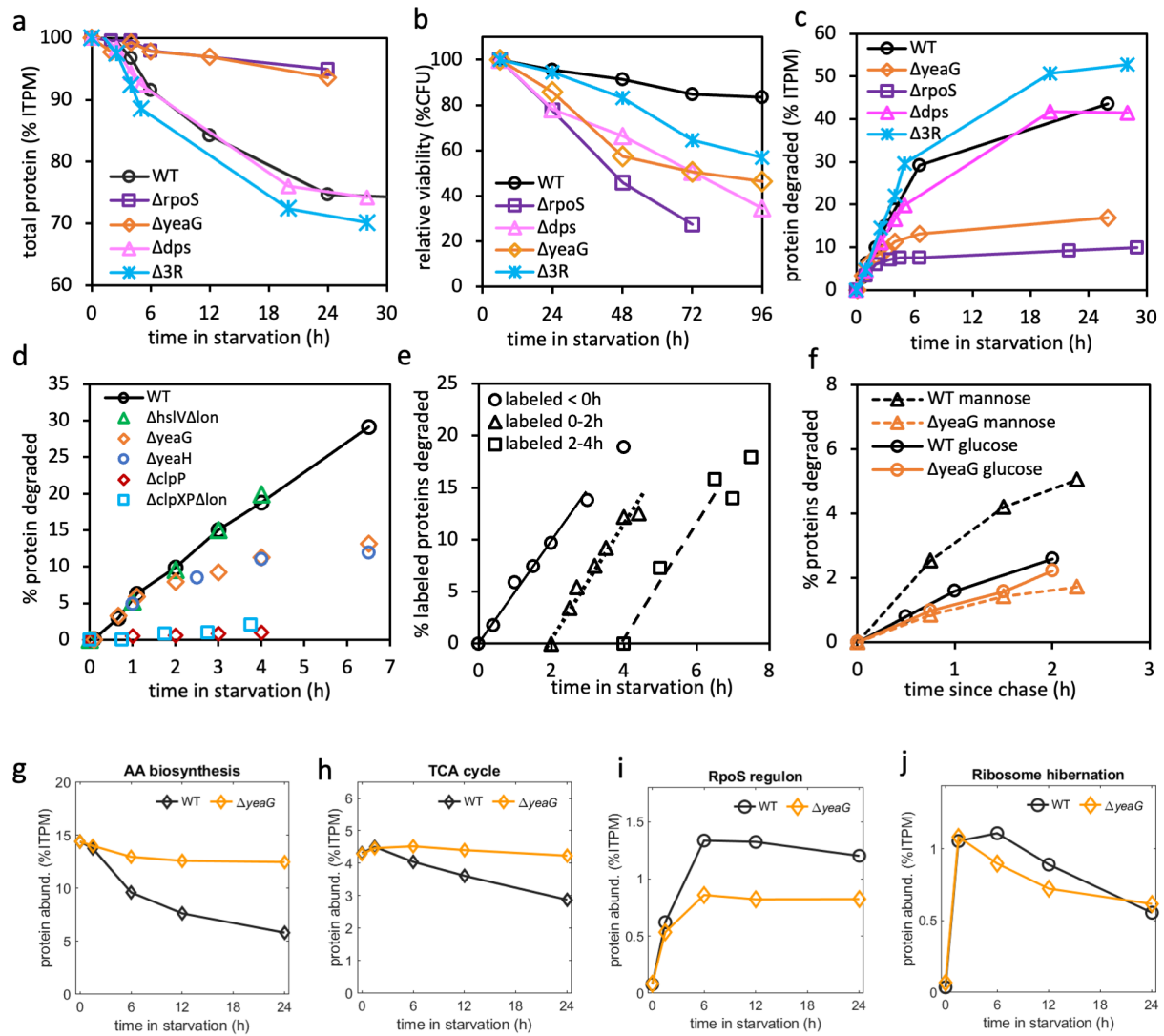
To identify this mediator, we individually deleted *dps* and *yeaG*, which are the most abundantly expressed proteins of the RpoS regulon after glucose depletion (Figure 3.Ab). $\Delta yeaG$ cells exhibited a marked reduction in protein loss, with total protein abundance similar to $\Delta rpoS$ cells and much smaller than WT cells (diamonds, Figure 4.Aa). In contrast, Δdps cells exhibited protein loss at similar level as WT cells (triangles, Figure 4.Aa), consistent with the known functional role of Dps in protecting the chromosome^{133,134}. As another control, we constructed a strain ($\Delta 3R$) deleted of the 3 hibernation factors (RaiA, Rmf, Hpf), two of which are very highly expressed in starvation (Figure 3.Ac). Protein loss exhibited by $\Delta 3R$ cells is similar to WT (Figure 4.Aa). In terms of viability, $\Delta yeaG$ cells exhibited substantial loss compared to WT cells (Figure 4.Ab), similar to the effects of deleting *dps*, reaching ~2/3 of the viability loss of $\Delta rpoS$ cells. This underscores the importance of YeaG to the survival of starving cells, possibly through its effect on protein loss.

To characterize the degradation process more directly, we adapted a radio-labelling method to quantify the extent of total protein degradation⁶⁷ (Appendix A3, Chapter 4 Methods). By pre-labeling proteins with ¹⁴C-leucine during exponential growth and

measuring its release during starvation, we found ~30% of labeled proteins degraded in WT cells within the first 6 hours of starvation (circles, Figure 4.Ac). $\Delta yeaG$ cells showed similar amount of degradation as WT cells during the first 2h, reaching 15% degradation by 6 h (diamonds, Figure 4.Ac). A dependence on YeaG is not expected the first 2h, since proteomic data showed low YeaG level in the first 1.5 h (Figure 3.Ab). From 2h to 24h however, the amount of protein degraded in $\Delta yeaG$ cells is 4-5x smaller than that of WT cells, with the latter reaching ~50% of the initial protein amount by 24h. Protein degradation in the other strains behaved similarly in agreement to their respective total protein loss (Figure 4.Aa), with Δdps cells and $\Delta 3R$ cells degrading similarly as WT cells and $\Delta rpoS$ cells similarly as $\Delta yeaG$ cells.

Figure 4.A: Characteristics of YeaG-dependent proteolysis

(a) Total protein amount remaining in the culture for $\Delta yeaG$ (diamonds), Δdps (triangles), and $\Delta raiA \Delta rmf \Delta hpf$ ($\Delta 3R$, asterisks) after glucose depletion. The data for WT and $\Delta rpoS$ cells are the same as in Fig. 1d. (b) Viability of the 5 cultures described in panel (a). (c) Direct measurement of protein degradation during glucose starvation. Proteins were labeled with ^{14}C -leucine during exponential growth and then chased with excess ^{12}C -leucine upon glucose depletion ($t=0$). The amount of ^{14}C -leucine in the supernatant reflects degradation of proteins synthesized before $t=0$; see Appendix A3. Plotted are the amount of pre-starvation proteins degraded for the 5 strains described in (a) and (b) during the first 24 hours of glucose starvation. (d) Same as (c), but for strains deleted of one or more proteases, or with the deletion of *yeaH*, which is co-transcribed with *yeaG* from the same operon. (e) ^{14}C -leucine was added for specific periods after glucose depletion (as indicated in the legend) to label proteins synthesized during those periods; see Appendix A3d. The culture was subsequently chased with excess ^{12}C -leucine, and the amount of ^{14}C -leucine released in the medium was recorded and plotted. The slope of the rise of each set of symbols from zero (various lines) indicates the degradation rate for the labeled proteins. (f) The rate of protein degradation during steady state growth is measured with minor modification as described in Appendix A3e. Measurements were taken for WT and $\Delta yeaG$ cells each grown in glucose and in mannose as the sole carbon source. The degradation rates, taken as the initial slope of the data points, is $\sim 1.2\%/h$ for WT cells growing on glucose (black circles), and similar for $\Delta yeaG$ cells in glucose or mannose medium (orange symbols), but ~ 3 -fold higher for WT cells grown in mannose (black triangles). This result is rationalized by the increase of YeaG protein levels in cells growing in poor carbon sources (such as mannose)⁸⁶. (g-j) Proteomic data for some exemplary functional groups expressed in WT and $\Delta yeaG$ cells, obtained as described in Figure 3.B and Figure 3.C.



4.2 Foray into YeaG mechanism and relationship to proteases

YeaG, thought to be driven by the nitrogen-responsive sigma factor RpoN (based on chip-seq data for NtrC binding^{141,142}) and investigated for its role in nitrogen starvation^{141,142}, possesses an N-terminal AAA+ ATPase domain^{142,143} and a C-terminal domain with distant homology to eukaryotic serine/threonine kinases¹⁴⁴ like its *Bacillus subtilis* homolog PrkA¹⁴⁵. Purified PrkA was shown to contribute to protein degradation *in vitro*¹⁴⁵, suggesting that it - and by extension YeaG - belongs to a large class of AAA+ ATPase "unfoldases" that play crucial roles in protein degradation in prokaryotes and eukaryote¹⁴⁶⁻¹⁴⁸. We generated point mutations of *yeaG* in both the AAA+ ATPase site (Walker B mutant E263A) and the putative kinase active site (K426A)^{142,146,149}, and found that two point mutants resembled the Δ *yeaG* mutant in both protein degradation and viability during starvation (Figure 4.B), suggesting that both domains are required for YeaG-mediated degradation. To identify additional requirements for YeaG activity, we found deletion of *yeaH*, the other gene in the operon with *yeaG*, reduced protein degradation to the same extent as Δ *yeaG* cells (Figure 4.Ad). Of the known cytoplasmic proteases¹⁵⁰, ClpP is required for proteolysis while the deletion of Lon and HslV shows little effect (Figure 4.Ad). Effort to delete all 3 proteases led to an unstable strain and will be pursued further elsewhere. Together, our data suggest YeaG to be an unfoldase which works together with ClpP and perhaps one of its partners to carry out the extensive degree of proteolysis observed.

Aside from the degradation of protein synthesized during exponential growth (Figure 4.Ac,d), we also characterized the degradation of proteins synthesized during starvation.

This is done by adding ^{14}C -leucine in the medium for a limited duration after glucose depletion and quantifying the subsequent release of leucine label (Appendix A3, Chapter 4 Methods). We find continued release of labeled leucine (Figure 4.Ae), indicating that the newly synthesized proteins are not stable themselves, and are degraded at rates even somewhat faster than those synthesized before starvation. YeaG is also found to enhance proteolysis during exponential growth. Although YeaG is hardly expressed for growth on glucose, it is expressed during growth on poor carbon sources such as mannose (along with other members of the RpoS regulon)⁸⁶. Accordingly, we find the rate of protein degradation to be reduced several folds for ΔyeaG cells grown in mannose but not in glucose (Figure 4.Af).

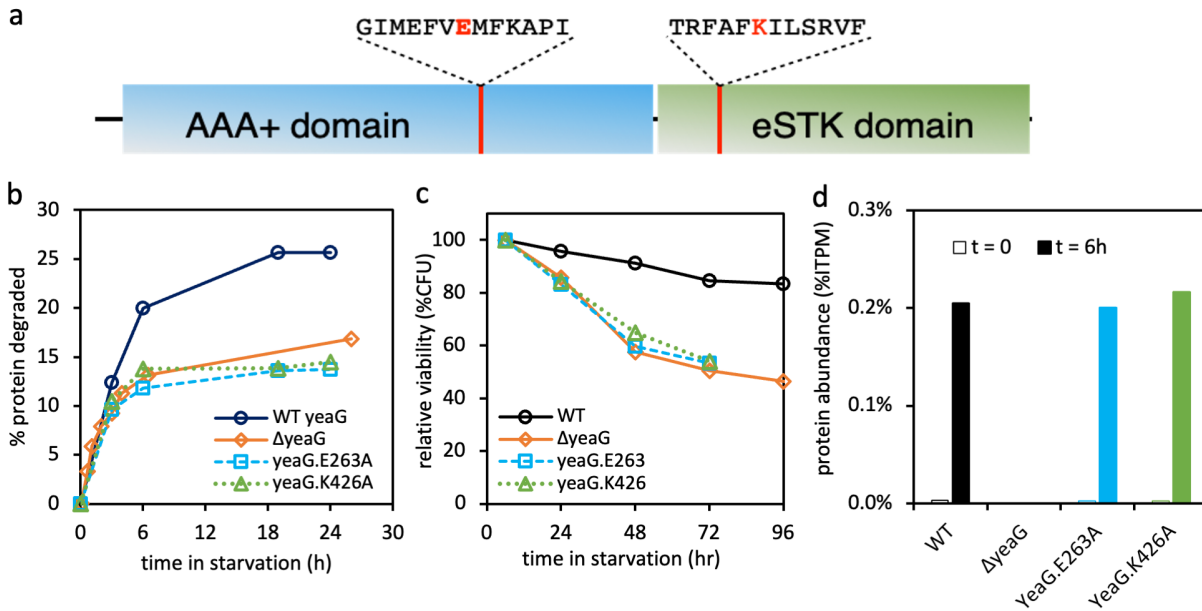


Figure 4.B: Genetic characterization of YeaG

(a) Graphical representation of YeaG's two putative protein domains: the N-terminal AAA+ ATPase domain associated with diverse cellular and the Eukaryotic-like Serine/Threonine Kinase (eSTK) domain^{142,143,151}. The Walker B (AAA+ domain) and eSTK functional motifs are annotated at their respective locations (red line) in each domain. Alanine mutations were made to known functional amino acids in each motif and are highlighted in red^{142,146,149}. Mutations E263A (gag to gcg) and K426A (aag to gct) were introduced into the *yeaG* gene driven by its own promoter and this construct was integrated into the native locus on the chromosome (see Chapter 2 Methods). (b, c) The extent of protein degradation and the relative viability during the first 24 hours of starvation for the *yeaG*.E263A (square, dashed line) and *yeaG*.K426A (triangle, dotted line) cultures. The data for WT and Δ yeaG are the same as those shown in Figure 4.Ab,c. (d) The amount of YeaG protein measured at 0h and 6h into starvation (open and filled bars) for the wild type strain, and the two point mutants: E263A, K426A (blue and green bars).

4.3 Analysis of Δ yeaG proteome

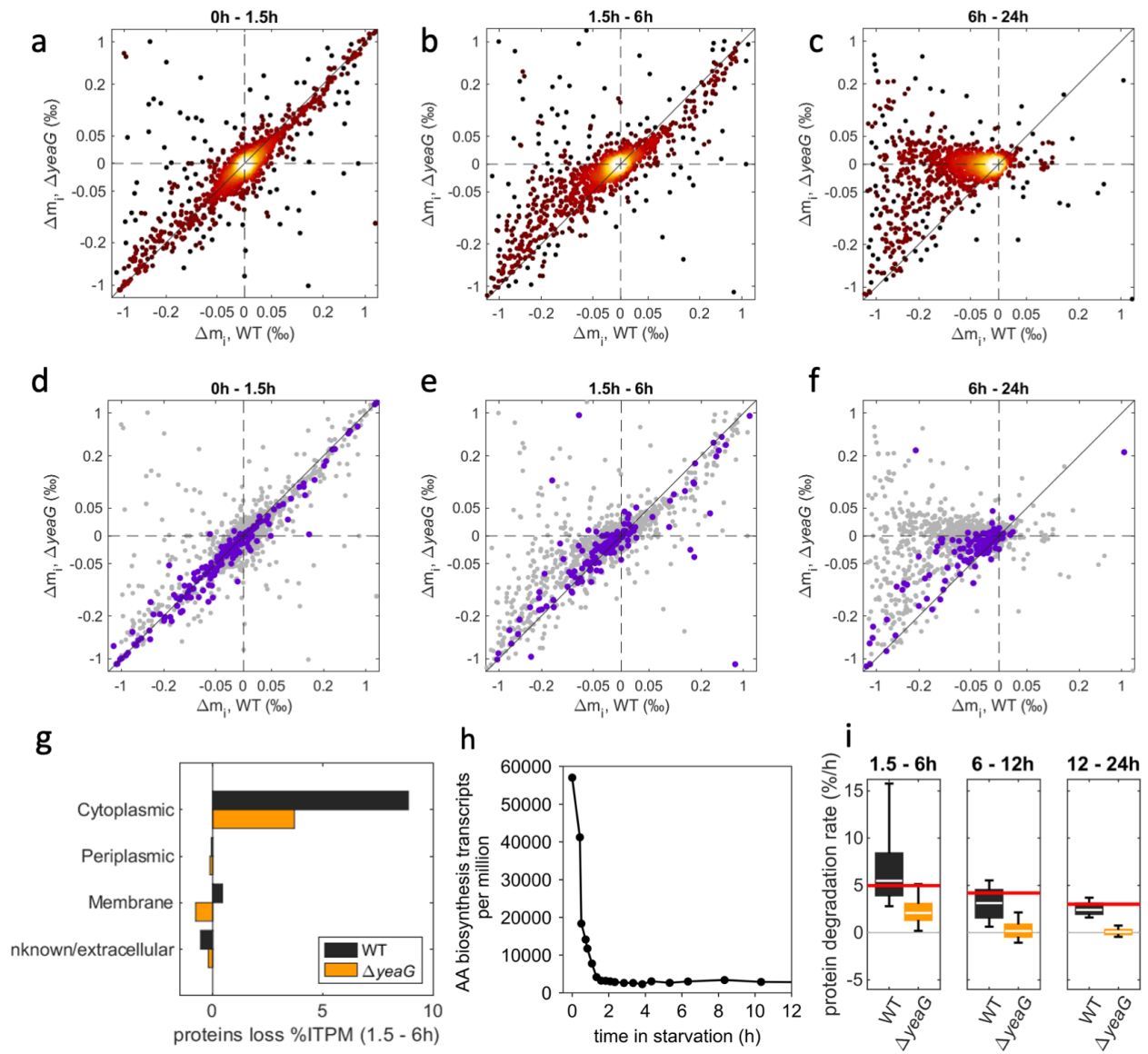
We repeated the proteomic analysis for Δ yeaG cells and compared its proteome dynamics with WT cells. Plotting changes in the proteome over different time intervals (Figure 4.Ca-c) showed that the proteome of both strains adapted similarly within the first 1.5h after starvation, again expected based on the low initial level of YeaG in WT cells. On the other hand, the abundance of many proteins was reduced after 1.5h in WT cells, but not in Δ yeaG cells, consistently with the observed overall decrease in proteolysis. Using available protein degradation rates recently measured during exponential growth¹⁵², we observe that yeaG deletion primarily affected proteins that are stable in exponential phase (Figure 4.Cd-f). We further observed that the dynamics of cytoplasmic proteins, but not periplasmic or cell envelope-associated proteins, was affected in the Δ yeaG strain compared to wild type (Figure 4.Cg). As AA biosynthesis enzymes are not synthesized during starvation as indicated by their low mRNA levels (Figure 4.Ch), the abundances of these proteins allowed us to estimate the kinetics of their degradation. We found widespread degradation in the WT strain, with typical rates $\sim 5\%/h$ before 6h into starvation, declining to $\sim 2.5\%/h$ within 24h (Figure 4.Ci); the degradation rates were strongly reduced in Δ yeaG cells. These data thus suggest that YeaG enables widespread degradation of cytoplasmic proteins during starvation.

To investigate how YeaG-mediated degradation affects viability (Figure 4.Ab), we further studied the proteome dynamics at the coarse level. As shown in Figure 4.Ca-e, we again observed a lack in protein remodeling after 6h into starvation. The lack of decrease of

proteins in biosynthesis and central carbon metabolism are particularly striking; two important sub-groups, AAB and TCA cycle, are shown in Figure 4.Ag, Figure 4.Ah, respectively. Looking further into the 4 groups that increased the most for WT cells (Figure 3.Ad), the gains by the RpoS regulon and ribosome hibernation proteins are diminished in $\Delta yeaG$ cells (Figure 4.Ai, 4j). Comparisons for exemplary members of these groups are shown Figure 4.Cf-l, with Dps and Rmf, two proteins that gained the most in WT cell, dropping by 50% or more in $\Delta yeaG$ cells. The reduced accumulation of these and other stress proteins (Figure 4.C) are likely responsible for the viability loss of $\Delta yeaG$ cells given their effects on viability (Figure 4.Ab).

Figure 4.C: Proteome comparison between WT and Δ yeaG cells

(a-c) Proteome adaptation to glucose depletion, measured as change in protein mass (%ITPM) for WT cells (x-axis) and Δ yeaG cells (y-axis) in different time intervals during starvation. To capture changes of different magnitudes and present both the positive and negative changes in a single plot, the data are shown in arc-tangent scale. (a) The initial adaptation (0 to 1.5h) is similar in the two strains (data close to the diagonal), consistent with the low level of YeaG expressed in WT cells (Fig. 2c). (b) Between 1.5h and 6h, differences are seen in the adaptation pattern as proteins tend to change less in Δ yeaG cells compared to WT cells. (c) After 6 hours, most of the proteins that decrease in abundance in WT show a lack of change in Δ yeaG strain. (d-f) Same data as panels (a-c), but highlighting proteins that are quickly turned over in exponential phase according to a recent study by Ref. [152]; high-lighted proteins have half-life <2 hours for cells growing in carbon-limited chemostat with 3h doubling times. Changes in the levels of these proteins are well correlated in the two strains, indicating that YeaG is not necessary for their degradation. Instead, YeaG enables degradation of proteins that are stable during exponential growth. (g) Protein loss (reduction in protein abundance) for proteins with different cellular localization (according to annotation provided by Ecocyc⁴⁴), between 1.5h and 6h from starvation. Cytoplasmic proteins are strongly reduced, particularly in the WT strain. The abundance of periplasmic and cytoplasmic proteins is much less affected during the same time interval. (h) According to a recent transcriptomics data of stationary *E. coli*¹⁵³, the mRNA levels of amino acid biosynthetic (AAB) enzymes dropped sharply in the first 1.5h and remained low through the course of glucose starvation. The plot here is obtained by summing the data of Ref. [93,153] for all genes belonging to the AAB group at each time point. (i) Due to their very low mRNA levels and hence the lack of synthesis (after the first 1.5h), the decrease in the abundance of the AAB proteins can be attributed primarily to protein degradation. Here, we estimated the degradation rate of each AAB protein i as $\ln[m_i(t_2)/m_i(t_1)]/(t_2 - t_1)$, where $m_i(t)$ is the mass of the protein measured by proteomics, and t_1, t_2 are two consecutive time points where data were taken. The degradation rates of WT are always larger than the Δ yeaG strain, and both are seen to decrease as the cultures enter deeper into stationarity. Horizontal white lines are medians. Filled boxes indicate 25-75% intervals. Whiskers indicate 10-90% intervals. For comparison, the red lines indicate the whole-proteome degradation rate for WT cells, obtained by dividing the protein degradation fluxes (dashed line in Fig. 4a) by the protein abundances measured at the beginning of each time window (Fig. 1d).



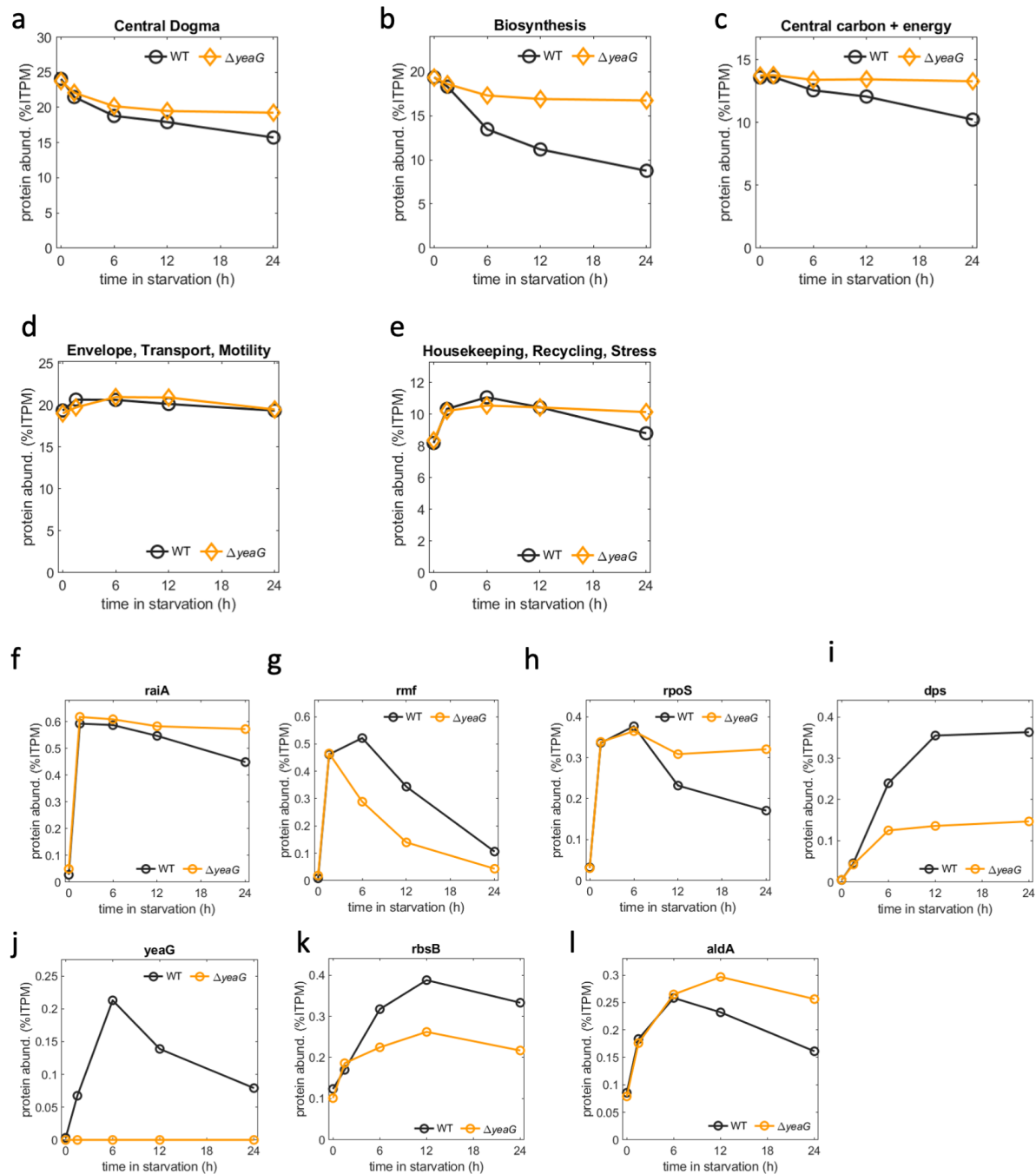


Figure 4.D: Comparison of proteome remodeling dynamics in WT and $\Delta yeaG$ cells

(a-e) Time courses for the total abundances of the five main protein functional categories for wild type and $\Delta yeaG$ cells in glucose starvation. Proteomics data was collected and processed in the same batch (see Chapter 3 Methods). (f-l) The dynamics of 7 proteins with the largest increase in protein abundance within 6 hours of starvation for wild type and $\Delta yeaG$ cells.

4.4 Methods

4.4.1 Instantaneous protein degradation rate

The scheme for measuring protein degradation rate is described in Appendix A3. 3 ml cultures were grown in the presence of 50 μ M ^{14} C-leucine [at 6.7 μ Ci/ μ mol] until glucose ran out. At the desired instant following starvation (for example, 6h, as illustrated in Appendix A3a) the external label was washed and chased by resuspending the cells in 3ml of MOPS buffer containing 1mM un-labelled leucine. 100 μ l culture was sampled every 30 minutes for the following 2 hours, precipitated with TCA as described above and the radioactivity in the TCA supernatant was monitored by scintillation. The rate of release of the label from proteins over short time intervals (2 hours) was taken as the instantaneous protein degradation rate. Minor modification of this procedure as described in Appendix A3d was carried out to measure the extent of degradation for proteins synthesized during starvation.

For measurement of protein degradation during exponential growth, minor alteration to the procedure was carried out as described in Appendix A3e. Cellular proteins were labelled with ^{14} C-leucine during exponential growth in minimal media containing either 20mM glucose or 3mM mannose as the carbon source as indicated in title. At OD₆₀₀ ~ 0.2, the label was chased by washing the cells with media containing the same carbon source, but devoid of ^{14} C-leucine and an excess of ^{12} C-leucine. Samples were collected and precipitated as described above.

4.4.2 Instantaneous protein synthesis rate

The scheme for measuring instantaneous protein synthesis rate is described in Appendix A4. 3ml cultures were grown in the presence of 50 μ M leucine until glucose ran out. At the desired instant following starvation (for example, 6h, as illustrated in Appendix A4a), 10 μ M ^{14} C-leucine [at 2 μ Ci/ μ mol] was pulsed in. 100 μ l cultures were sampled every 30 minutes for the following 2 hours, precipitated with TCA and the radioactivity in the precipitate was monitored as described earlier to measure the amount of leucine incorporated into protein. This amount of incorporated leucine was compared to the measured leucine content of protein during growth (265 μ M/OD, Appendix A4b) to express synthesized protein as a percentage of the protein content during growth.

4.5 Acknowledgements

With permission from co-authors, this chapter reproduces materials and analysis, in part, from the following paper in preparation: Manzanarez, Gabriel[†]; Balakrishnan, Rohan[†]; Kannan, Harish[†]; Abele, Miriam; Zhang, Zhongge; Wu, Chenhao; Freiman, Lance; Enuston, Eray; Corbett, Kevin D.; Ludwig, Christina ^{*}; Mori, Matteo ^{*}; Hwa, Terence^{*}. “YeaG-mediated global proteolysis in starving bacteria dictates protein synthesis and prolongs cell survival”. The dissertation author was the primary author of this paper.

Chapter 5: Flux coordination between protein synthesis and degradation

5.1 Relationship between protein synthesis and degradation

Why would the deletion of YeaG, a mediator of proteolysis, result in reduction of stress proteins such as Dps, particularly when the RpoS protein itself is increased in Δ yeaG cells (Figure 4.C)? This question was raised long ago¹⁵⁴ when a similar effect was found upon the deletion of *clpP*. Within our resource allocation framework (Figure 2.Ac), the answer lies generally in the reliance of protein degradation to generate resources for protein synthesis. To probe the validity of this framework, we next measure directly the protein degradation and synthesis fluxes and characterize their mutual relationship over the course of starvation.

The radio-labeling method is modified to measure the “instantaneous” flux of degradation at different time after starvation (Appendix A3a-c, Chapter 4 Methods). As shown in Figure 5.Aa, the degradation flux sharply increased from the steady state value (dashed line) upon entering starvation for both WT and Δ yeaG cells; this initial increase is YeaG-independent, possibly resulting from a general problem with protein quality as the cytoplasm condenses shortly after glucose depletion.^{155,79} While the degradation flux of Δ yeaG cells (diamonds) drops sharply after 1-2 hours towards the basal level (dashed line), it is maintained in WT cells (circles) for the first 5-6 hours before declining gradually over the course of the next 20 hours. This data is consistent with the qualitative picture derived from

the losses of pre-shift proteins measured in Figure 4.Ac, but additionally provides a quantitative temporal measure of the boost in proteolysis due to YeaG.

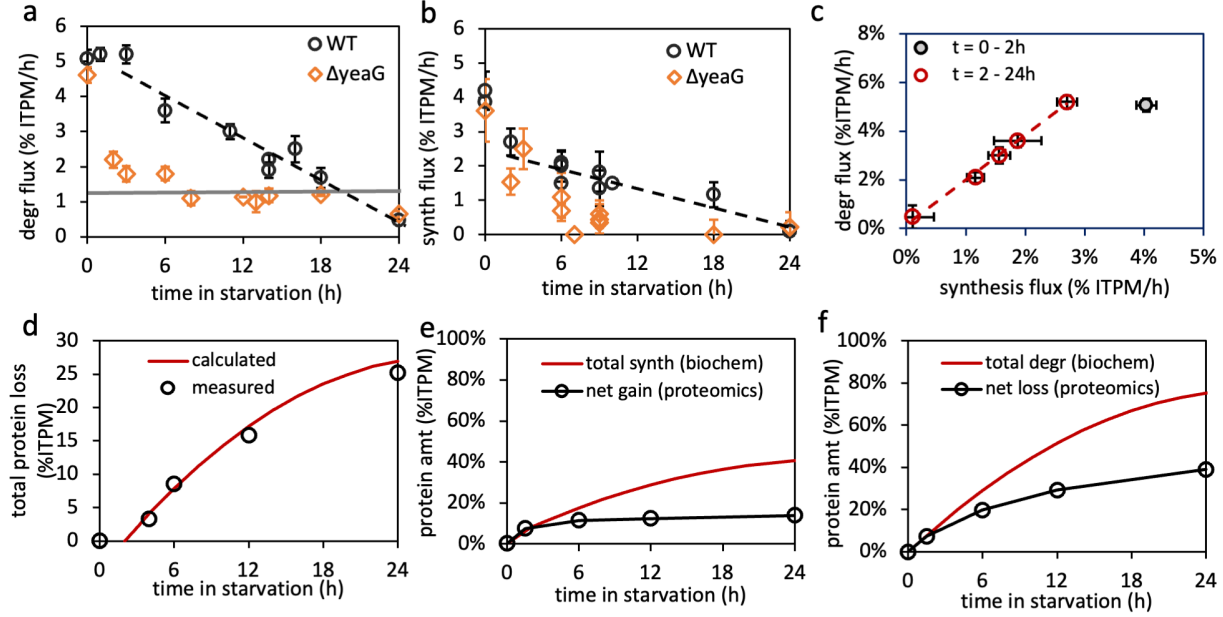


Figure 5.A: Flux of protein synthesis and degradation

(a) The flux of protein degradation in a culture at various time t after glucose depletion, $J_D(t)$, obtained by measuring the amount $\Delta M(t)$ of ^{14}C -leucine released into the medium over the course of a period Δt (about an hour) after cells grown in ^{14}C -leucine is chased by the addition of excess ^{12}C -leucine at time t ; see Appendix A3. The degradation flux at time t is taken to be $\Delta M(t)/\Delta t$. Circle: WT cells; diamonds: $\Delta yeaG$ cells. Dashed line: degradation flux during exponential growth (from Fig. 3f). (b) The flux of protein synthesis at various time t after glucose depletion, $J_S(t)$, is obtained by adding ^{14}C -leucine to the stationary culture and measuring the depletion of radioactivity in the medium; see Appendix A4. Dashed lines in panels (a) and (b) are the best linear fits to the data for WT cells, excluding the data at $t = 0$: $J_S(t) = 2.56 \cdot (1 - t/25.9h)$ and $J_D(t) = 5.27 \cdot (1 - t/26.2h)$. Vertical bars in panels a, b represent technical errors (Appendix A3). (c) The data for WT cells in panels (a) and (b) are partitioned into 6 bins, and the average degradation flux of each bin is plotted against the average synthesis flux of that bin. Except for the first bin (0-2h, filled circle), the data from the other 5 bins exhibit a linear relation (dashed line) with slope 1.83 ± 0.1 and y-intercept $0.16 \pm 0.17\%$ ITPM/h. Error bars: SEM within bin. (d) Total protein loss, computed as $\Delta M_{loss}(t) = \int dt' [J_D(t') - J_S(t')]$ (red line), compared to the measured data taken from Figure 2Ad (circles). (e) Amount of protein synthesized, computed as $\Delta M_{synth}(t) = \int dt' J_S(t')$, (red line), compared to the data from proteomics (circles, Fig. 2f). (f) Amount of protein synthesized, computed as $\Delta M_{degr}(t) = \int dt' J_D(t')$, (red line), compared to the data from proteomics (circles, Fig. 2g).

We also reversed the radio-labeling protocol to obtain the “instantaneous” synthesis flux for the first 24h after starvation (Appendix A4, Chapter 4 Methods). As seen in Figure 5.Ab, the synthesis flux of $\Delta yeaG$ cells dropped rapidly compared to that of WT cells a few hours into starvation. However, a more quantitative comparison is prevented by the resolution of our measurements ($\sim 0.5\%$ ITPM/h). We next compared the synthesis and degradation fluxes for WT cells, denoted as $J_S(t)$ and $J_D(t)$, respectively, which both decrease over time (circles, Figure 5.Aa, Figure 5.Ab). In the first 1-2h, the two fluxes were quite similar, but a gap occurred between the two fluxes subsequently. A scatter plot of the two fluxes (Figure 5.Ac) reveals a simple linear relation between them except for an aberrant point associated with the first hours of starvation (arising from a small amount of overflow acetate in the medium, as explained in Figure 5.B).

The linear relation, written as

$$J_D(t) = (1 + \eta) \cdot J_S(t) + J_0 \quad (1)$$

has a slope of ~ 1.8 , indicating that on average 1.8 proteins are degraded for every protein synthesized, a surcharge of $\eta \approx 80\%$ for protein synthesis through the first 24h of starvation. This surcharge is consistent with the minimum surcharge of 50% needed to generate ATP from AA catabolism described above. If we interpret this 80% surcharge as going completely into the energy need of protein synthesis, then at 4 ATP/peptide bond, it implies that $4/\eta \approx 5$ ATP can be generated for every AA catabolized, which is $\sim 60\%$ of the maximum conversion efficiency of ~ 8 ATP/AA predicted by FBA (Figure 2.Dc). This level of reduction from the maximum ATP conversion efficiency is not surprising given that the

NADH-ATP conversion is not expected to be maximal⁹² (Table 2.3) and not all AA recycling pathways are expressed (Figure 2.Dc).

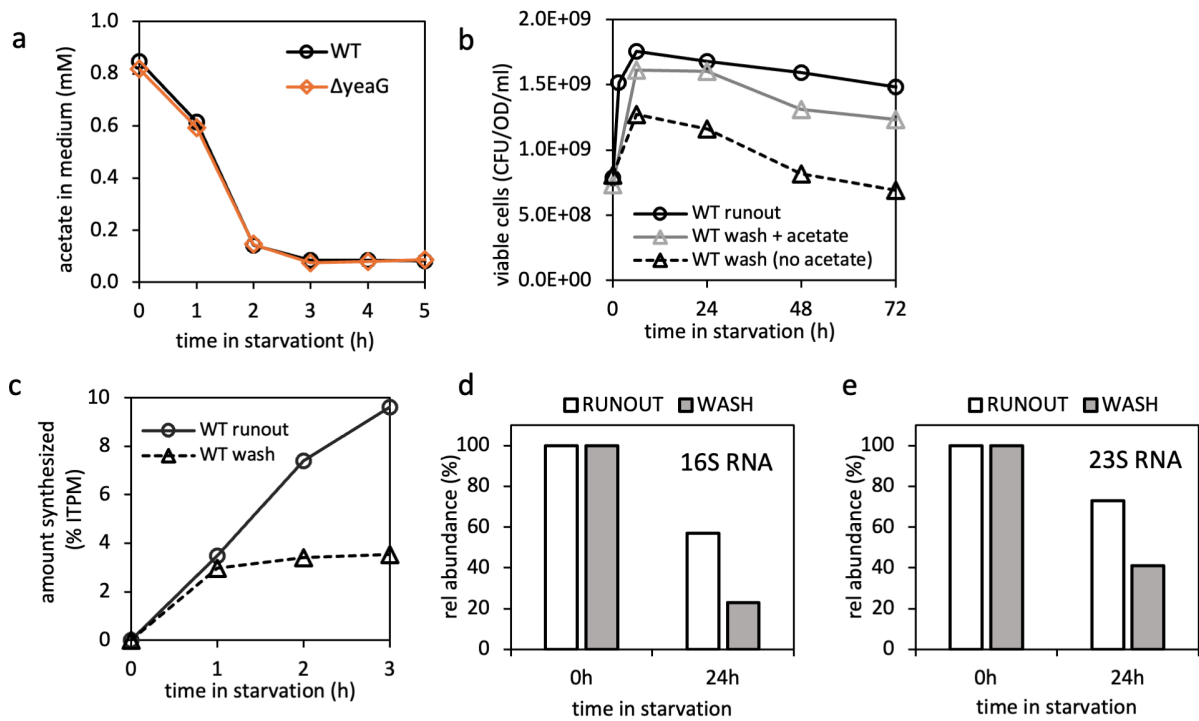
Another feature of the data in Figure 5.Aa, Figure 5.Ab is the approximately linear time-dependence of the synthesis and degradation fluxes (dashed lines). These linear forms allow us to compute the expected total protein loss, which compares very well with the independently measured protein loss (line and circles, Figure 5.Ad), thereby validating both measurements. Additionally, we compared the total proteins synthesized by the culture after a period of time t in starvation, obtained from the time-integral of $J_S(t)$, to the net proteins gain at time t obtained from proteomic data (Figure 3.Af). Figure 5.Ae shows that the protein gained at 24h of starvation is only $\sim 1/3$ of the proteins synthesized. This indicates a high degree of futile synthesis, i.e., $\sim 2/3$ of the protein synthesized ($\sim 30\%$ ITPM) were degraded, as reflected by a similar gap in the amount of proteins degraded and the net protein loss (Figure 5.Af). The high degree of futile synthesis is expected mechanistically based on the high degradation rate of proteins synthesized during starvation (Figure 4.Ae) and the non-specific nature of YeaG-dependent degradation (Figure 4.Ci). Thus, the combined effect of the energy surcharge for protein synthesis and the futility of synthesis due to persistent degradation makes it very costly to accumulate desired proteins in starving cells, requiring the turnover of 5-6 proteins to gain one protein by 24h of starvation.

Another important feature of the linear relation in Figure 5.Ac is the y-intercept, or the term J_0 in Eq (1), which is independent of the extent of protein synthesis and can be interpreted as the flux of degraded proteins used to supply the maintenance energy, i.e., an

ATP maintenance flux, $J_M = (4/\eta) \cdot J_0$. The value of the intercept gives $J_M \leq 0.1$ mmol ATP/gCDW/h, which is $\sim 30\times$ smaller than the maintenance flux extrapolated from exponential growth^{156,92}. The smallness of this maintenance flux compared to the ATP flux for protein synthesis ($4 \cdot J_S$) during the first 24h shows that protein degradation in this period is almost exclusively geared towards protein synthesis.

Figure 5.B: The effect of overflow acetate on starvation response and viability

(a) Acetate in culture media after removing cells via filtration at various time after 2mM glucose runs out at OD $\gg$ 0.4 ($t=0$, Figure 2.Aa) for WT and $\Delta yeaG$ cultures. 0.86mM of acetate in the medium at the onset of starvation (due to acetate overflow, $\sim 2\text{mM}/\text{OD}^{76}$) is taken up in the subsequent 2 hours. This acetate taken up presumably relieved the need for an increased degradation flux compared to synthesis flux as seen in the first 1-2 h of starvation (Figure 5.Ac). This amount of overflow acetate turns out to be important for the physiology of the starving cells, as we show in the next panels by comparing the “runout” treatment studied for most of this work with the “washout” treatment. Here, “washout” refers to pelleting growing cultures at OD600 $\sim$ 0.4 and resuspending the pellet in fresh medium with no glucose or acetate; see Methods. (b) The viability of the cells clearly suffered in the washout treatment compared to runout (solid and dashed line, respectively), both in the first several hours where the rise due to reductive division is reduced after washout, and after 3 days if one compares to viable cell count to the peak count at 6h. Most of this viability loss by the washout treatment can be rescued by adding back the 0.86mM acetate when washed cells are resuspended with fresh medium (grey line), suggesting that the difference in viability resulted from some long-term effect exerted by the acetate taken up in the first 2h. Possible effects include the use of acetate to fuel the synthesis of stress proteins, and the storage of acetate as fatty acid (used latter to supplement cell maintenance). (c) Direct measurement of protein synthesis (Appendix A4) shows that protein synthesis is drastically reduced for cells subjected to the washout treatment. (d,e) Measurements of the full-length 16S and 23S rRNA by TapesStation (see Figure 2.F) show that the ribosomes are substantially disrupted for cells subjected to the washout treatment, with only 20% of its 16S rRNA intact 24h after washout, indicating greatly diminished translational machinery under this treatment. Altogether, the data in panels b-e indicate that the uptake of overflow acetate in the first 2h drastically affects the starvation response. Therefore, we focus our effort in this study to characterize starvation resulting from runout but not washout.



5.2 Strategy of flux coordination

The tight linear relation between synthesis and degradation fluxes (Figure 5.Ac) raises the question of how the two are coordinated. There exists in principle two strategies of coordination [scott23 NRM]: A demand-driven strategy where degradation is set by the demand of energy, in this case, the need for protein synthesis (Figure 5.Ca). An opposing strategy is supply-driven, in which synthesis is set by the amount of proteolysis (Figure 5.Cb). These two strategies can be distinguished by perturbing the ATP pool: An injection of energy would inhibit protein synthesis in the demand-driven strategy but would increase synthesis in the supply-driven strategy.

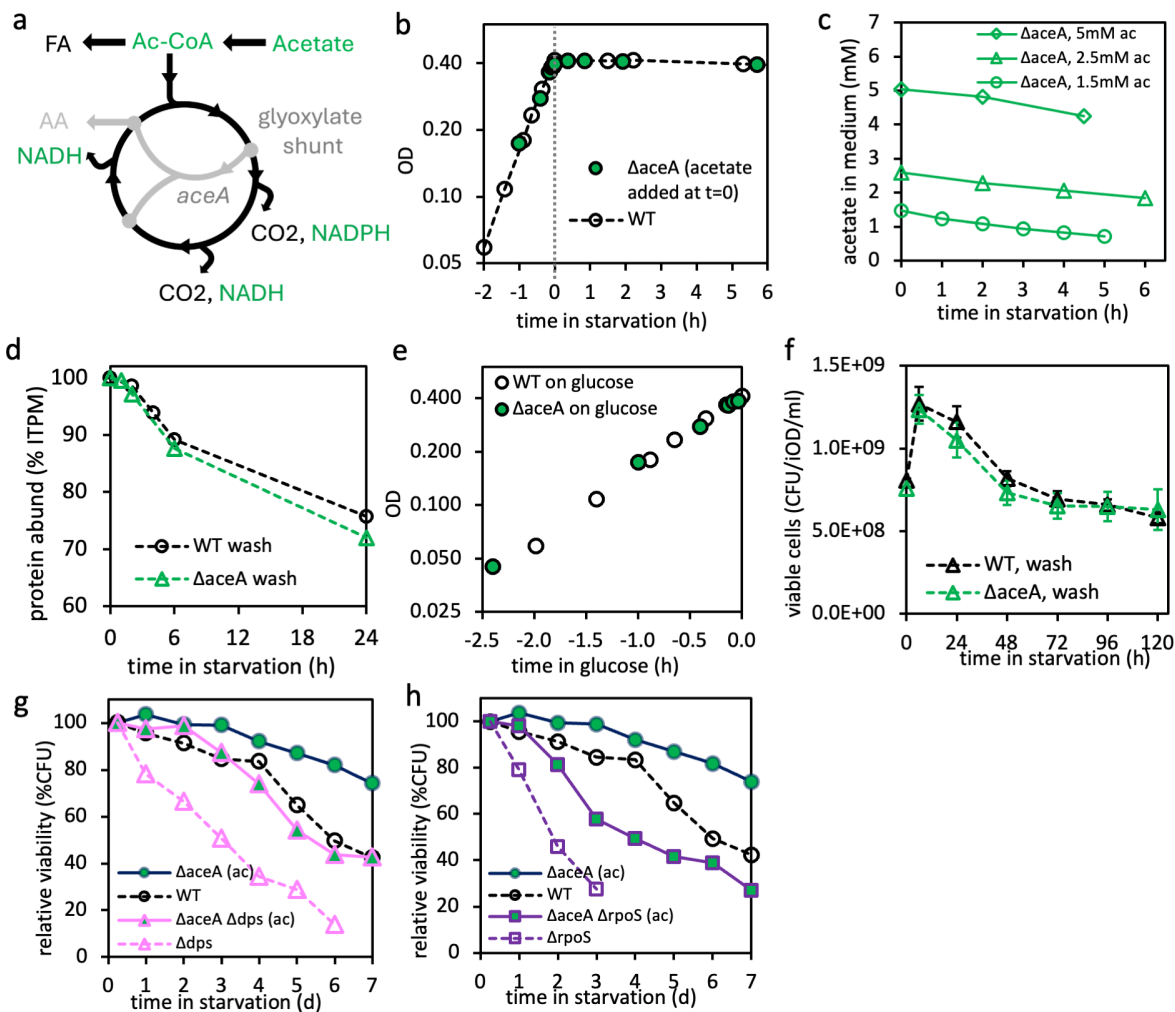
We devised an approach to implement this energy perturbation: As described in Figure 5.Da, the glyoxylate shunt is required for biomass generation when *E. coli* grows on acetate as the sole carbon source^{157,158}. By deleting *aceA* which is one of the genes encoding the glyoxylate shunt¹⁵⁹, *E. coli* can use acetate for energy generation via the TCA cycle or convert it to fatty acid, but cannot generate most of the biomass components necessary for growth. Accordingly, $\Delta aceA$ cells cannot grow on acetate as the sole carbon source (Figure 5.Db), but acetate is still consumed slowly (Figure 5.Dc), presumably to generate energy.

Figure 5.C: Coordination of protein synthesis and degradation

(a,b) Illustrations of two strategies of flux coordination. In the demand-driven strategy, depletion of energy (the box filled in green) due to the demand of synthesis removes the inhibition of proteolysis. In the supply-driven strategy, increase in energy due to increased proteolysis enables increased protein synthesis. The two strategies can be distinguished by perturbing the energy pool (the open green arrow) and observing whether the response is dominated by changes in the synthesis or degradation flux. Perturbation of the energy pool can be implemented by supplementing acetate to cells deleted of *aceA*, as acetate can be used to generate energy but not for biomass growth; see Figure 5.D. (c) $\Delta aceA$ cells were grown in glucose MOPS medium. When the exponentially growing culture reached OD = 0.4, cells were washed and resuspended in fresh MOPS medium without any carbon source ($t = 0$). 6h later, the culture was either supplemented with 5 mM acetate (filled symbols) or no acetate (open symbols). Total protein content in each tube was monitored through 24h. (d) In the experiment described in panel (f), protein synthesis flux was measured as described (panel b and Appendix A4) for the two cultures of $\Delta aceA$ cells with and without acetate supplement at 6h after glucose depletion, with ^{14}C -leucine added at $t = 6h$. The amount of synthesis, taken as the reduction of radioactivity in the medium, was measured and plotted for the subsequent 45 min. (e) WT cells were grown exponentially in ^{14}C -leucine and entered glucose starvation as described in Fig. 1a. 6h after starvation, the culture was chased with excess ^{12}C -leucine with and without the addition of 35 μM of Chloramphenicol (filled and open symbols, respectively). The amount of radioactivity in the medium, taken as the amount of protein degraded (Appendix A1) was monitored and plotted for the subsequent 45 min. (f) Total protein abundances (Figure 4.Aa, Figure 4.A) were measured for WT and $\Delta yeaG$ cultures for up to 6 days after glucose starvation. The initial rapid total protein loss of the WT culture (25% in 1d, dotted black line) stopped abruptly after the first day, followed by a much slower loss of 1.3%/d (dashed black line). The low degradation flux after day 1 is of the magnitude of the offset J_0 in Eq. (1) which may reflect the energy flux demanded by cell maintenance. The $\Delta yeaG$ culture does not have the big initial protein loss as already discussed; however, it exhibits a 2-fold larger long-term loss rate ($\sim 2.5\%/d$, dashed orange line).

Figure 5.D: $\Delta aceA$ strain and acetate supplement

(a) Diagram indicating key acetate-utilizing metabolic pathways, including energy generation via the TCA cycle and oxidative phosphorylation, fatty acid (FA) biosynthesis, and gluconeogenesis via the glyoxylate shunt (grey text, and arrows). Anabolism of AA via the glyoxylate shunt encoded by *aceA* and *aceB*. Expression of both genes is repressed during growth on glucose and turned on during the first hours of glucose starvation²¹. We deleted *aceA* to remove the cell's capacity to synthesize AAs from acetate, such that acetate can be used as an energy source (and possibly be stored as fatty acids), but not for cell growth. (b) OD600 of WT and $\Delta aceA$ cultures, which grew exponentially on glucose and entered starvation via glucose depletion shown in Figure 2.Aa. The $\Delta aceA$ culture did not grow even when supplemented with 5mM acetate after glucose depletion, validating the expected inability to grow on acetate. (c) Varying concentrations of acetate were added to the $\Delta aceA$ culture after glucose depletion. Acetate was seen consumed by the culture despite its lack of growth. The rate of consumption was similar for acetate concentration at 1-5 mM, indicating that the uptake is not concentration limited. In all subsequent experiments involving acetate-supplemented $\Delta aceA$ strains (including those in combination with other mutations studied, such as $\Delta yeaG$, Δdps , or $\Delta rpoS$), the concentration of acetate was routinely monitored and additional supplementations made as needed such that acetate concentrations always remained above 1mM. (d, e, f) In the absence of acetate supplement, WT and $\Delta aceA$ cells behaved similarly: They grew similarly on glucose (panel e), and after washed into fresh medium without glucose or acetate, the viability dropped similarly (panel f) and also suffered similar protein loss (panel d). (g) Viability of $\Delta aceA \Delta dps$ cells supplemented with acetate (ac) (filled triangles) is substantially higher than that of Δdps cells (open triangles, same data as that shown in Figure 4.Ab), but lower than $\Delta aceA$ cells supplemented with acetate (filled circles, same data as that shown in Figure 6.Ab). The data indicates that energy supplement can partially rescue the deleterious effect of *dps* deletion. (h) Viability of $\Delta aceA \Delta rpoS$ cells supplemented with acetate (ac) (filled squares) exhibits higher viability that of $\Delta rpoS$ cells (open squares, same data as that shown in Figure 4.Ab), but lower than $\Delta aceA$ cells supplemented with acetate (filled circles, same data as that shown in Figure 6.Ab). Thus, again energy supplement can partially rescue the deleterious effect of *rpoS* deletion. (i) Viability of $\Delta aceA$ cells supplemented with acetate (ac) and with translation inhibited by the addition of chloramphenicol at different times during early starvation. Taken together, panels (g-i) indicate that RpoS-regulon gene expression is important for viability even in the presence of energy supplementation, but only during the first two hours of starvation. Protein synthesis after 2h is not needed for viability in the presence of energy supplementation.



ΔaceA cells, when depleted of glucose, exhibit protein loss similar to that of WT cells (Figure 5.Dd) When 5mM of acetate is added to the culture 6h into glucose starvation, the loss of proteins is abruptly arrested (filled circles, Figure 5.Cc), reflecting the immediate adjustment of the difference of the degradation and synthesis fluxes when the derivation of energy from AA catabolism is relieved. This adjustment could involve reduction in degradation (demand-driven strategy) or increase in synthesis (supply-driven strategy). Direct measurement in Figure 5.Cd shows an immediate increase of the synthesis flux, by 2~2.5%ITPM/h, comparable to the change in the protein loss flux of ~2.1%ITPM/h seen in Figure 5.Cc. The result thus supports the supply-driven strategy of flux coordination (Figure 5.Cb). The recovery of synthesis flux upon energy supplementation requires a mechanism for allocation of AA from protein turnover towards ATP vs protein synthesis; this can be largely accomplished by mass action alone (Figure 6.C).

As a separate test of the coordination strategy between synthesis and degradation, we added chloramphenicol to the culture of WT cells 6h into glucose starvation. The abrupt stoppage of protein synthesis, hence the abrupt relief of the energy demand, would lead to an arrest of protein degradation in the demand-driven strategy (Figure 5.Ca). Instead, protein degradation only reduced by 10~20% in the next 45 min (Figure 5.Ce), again supporting the supply-driven strategy. Together, these results indicate that not only is YeaG-mediated proteolysis used to fuel protein synthesis, but the amount of this degradation sets the amount of protein synthesis possible.

Interestingly, YeaG-mediated proteolysis appears to stop abruptly after the first 24h (Figure 5.Cf), which matches with the behaviors of the measured synthesis and degradation fluxes (Figure 5.Aa,b). As the effect of YeaG on viability extends for days after (Figure 4.Ab), these data suggest YeaG-mediated proteome remodeling is a costly but critical investment starving cells make early on to improve their long-term viability.

5.3 Methods

5.3.1 Acetate measurement

The concentration of acetate in the extracellular medium was quantified using HPLC (Shimadzu LC-20AB solvent delivery unit connected to a Shimadzu RID-20A refractive index detector). 150µl of culture samples was filtered via centrifugation at 9K rpm through a 0.22µm filter for 30 seconds and stored at -20°C before performing HPLC. The solvent was 0.01 M H₂SO₄ with a flow rate of 0.4 mL/min. HPLC peaks were calibrated using python code created in the lab and peak intensities calibrated to samples with standards of known concentrations.

For acetate uptake under acetate supplementation, sodium acetate was supplemented in the media at regular intervals to consistently maintain an acetate concentration above 1mM. Uptake rates were calculated at by linear regression through 3 or more samples taken at 1–3-hour intervals around each timepoint, depending on how rapidly the uptake rates were changing at that timepoint, for example during earlier timepoints, when the uptake rates were changing rapidly, sampling was done more frequently, every hour or less.

5.4 Acknowledgements

With permission from co-authors, this chapter reproduces materials and analysis, in part, from the following paper in preparation: Manzanarez, Gabriel[†]; Balakrishnan, Rohan[†]; Kannan, Harish[†]; Abele, Miriam; Zhang, Zhongge; Wu, Chenhao; Freiman, Lance; Enuston, Eray; Corbett, Kevin D.; Ludwig, Christina *; Mori, Matteo *; Hwa, Terence*. “YeaG-mediated global proteolysis in starving bacteria dictates protein synthesis and prolongs cell survival”. The dissertation author was the primary author of this paper.

Chapter 6: Effect of global proteolysis on long-term viability

We now turn to the physiological roles of YeaG-fueled protein synthesis on cell viability. The protein synthesized, including the RpoS regulon, ribosome hibernation factors, and recycling enzymes catalyzing the catabolism of AA, nucleotides, and fatty acids (Figure 3.B, Figure 3.C) can contribute to extending cell survival by a variety of means (Figure 6.Aa). Qualitatively, the effects of these factors belong to two classes, those that protect essential cellular components and those that reduce the cost of cell maintenance. For example, Dps compacts the chromosome and presumably¹³³ protects it from damage, while ribosome hibernation factors protect the ribosome from degradation⁷⁵. The recycling enzymes instead reduce the maintenance demand by increasing the conversion of biomass components to ATP (Figure 2.D, Figure 2.E). Additionally, global proteolysis could reduce enzymes involved in futile cycling and thus further reducing maintenance.

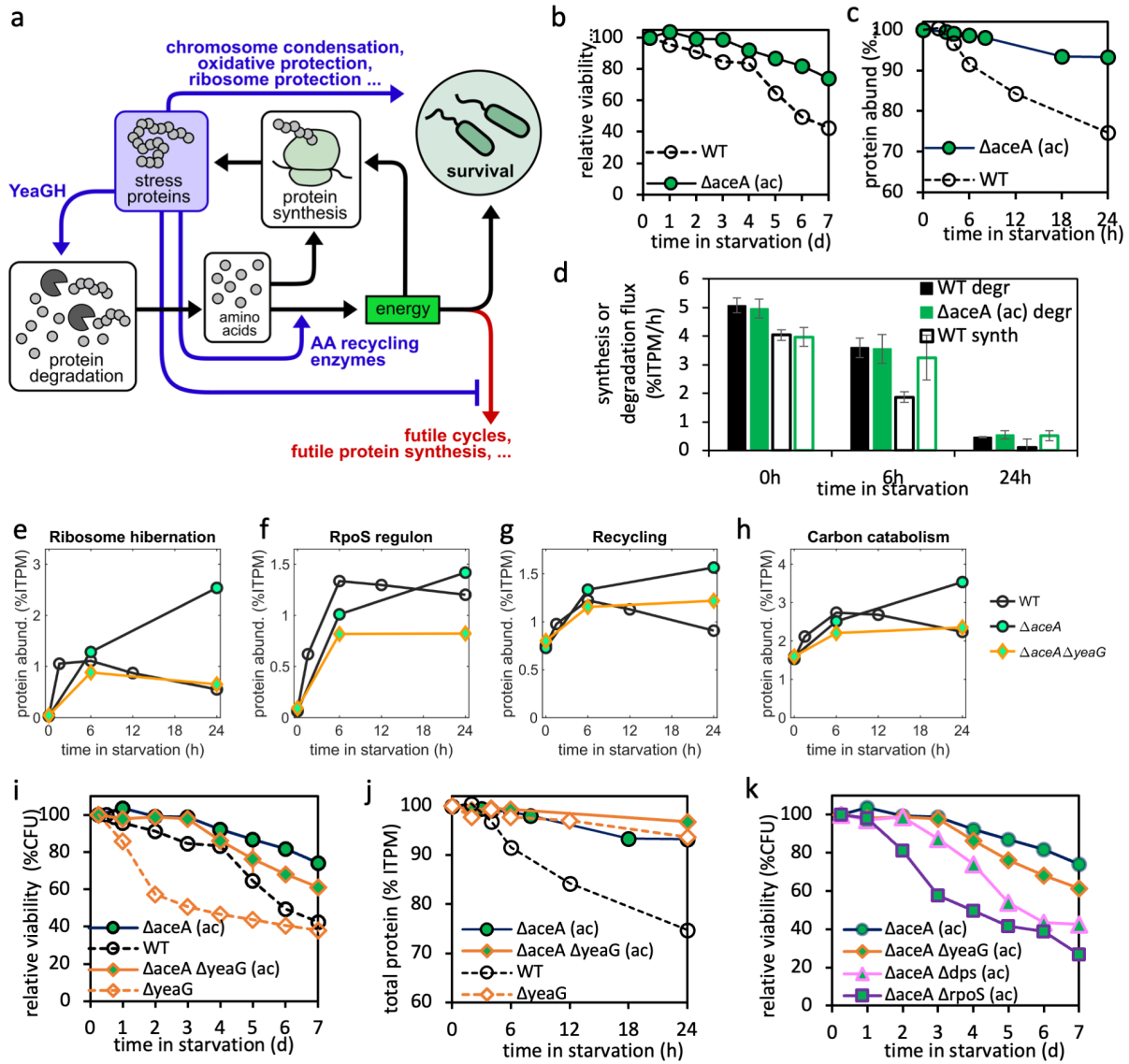
6.1 Effect of energy supplementation on the viability of WT cells

It is possible to distinguish these two classes of effects by comparing their contributions to long-term viability during complete carbon-starvation vs glucose starvation with energy supplement, with the latter implemented by providing external acetate to $\Delta aceA$ cells (Figure 5.Da). In the absence of acetate supplement, $\Delta aceA$ cells grows similarly as WT and their viability drops similarly as WT (Figure 5.De,f) With supplement that maintains acetate at several mM in the medium, the viability of $\Delta aceA$ cells is clearly improved over that of WT cells (Figure 6.Ab). Further, protein loss is reduced in $\Delta aceA$ cells (Figure 6.Ac), consistent with the relief of protein turnover for energy biogenesis (Figure 2.Ac) when energy

is provided, as well as the transient rescue of protein loss after acetate addition (Figure 5.Cc). The lack of protein loss in energy supplemented cells resulted not from reduction in degradation but from increase in synthesis (Figure 6.Ad), again consistent with the supply-driven strategy of flux coordination (Figure 5.Cb).

Figure 6.A: Effects of energy supplement and protein synthesis on long-term viability.

(a) Overview of the physiological functions of “stress proteins” synthesized during starvation: Some play a protective role (upper purple arrow), others serve to support energy maintenance (lower purple arrows), either by improving the efficiency of ATP conversion or by reducing the cost of maintenance (e.g., by reducing futile cycles). Only the conversion of AA to energy is shown but fatty acids and nucleotide also contribute. (b) Viability of glucose starved $\Delta aceA$ cells (filled circles), with excess acetate (5mM acetate added at the time of glucose depletion ($t=0$) and further addition as needed at later times to maintain acetate concentration above 1mM in the medium throughout the duration of the experiment; see Figure 5.D. Viability data for WT cells (open circles) is the same as that in Figure 2.Ba. (c) Total protein data for $\Delta aceA$ cells (filled symbols) were taken as described for WT cells (open symbols) in Appendix A1, A2, but with 5mM acetate added at $t=0$ such that acetate remained >1 mM at $t=24$. (d) Degradation and synthesis flux of WT (filled and open black bars, respectively) are taken from the data shown in Figure 5.Aa,b. Degradation flux of $\Delta aceA$ cells (filled green bars) was measured relative to that of WT cells in parallel for each time point. Synthesis flux was estimated as the difference of the degradation flux at each time point and the protein loss rate at the corresponding time point (total protein content two hours apart). (e-h) Abundance of the 4 protein groups that increased in WT cells (open circles) as shown in Figure 3.Ad, but also including $\Delta aceA$ cells (filled circles) and $\Delta aceA \Delta yeaG$ cells (filled diamonds), each supplemented with excess acetate starting $t=0$. (i) Same as (b), but including $\Delta aceA \Delta yeaG$ cells supplemented with acetate starting $t=0$ (filled diamonds). Viability of $\Delta yeaG$ cells (open diamonds) is the same as that shown in Figure 4.Ab. (j) Same as (c), but including $\Delta aceA \Delta yeaG$ cells supplemented with acetate from $t=0$ (filled diamonds). Total protein of $\Delta yeaG$ cells (open diamonds) is the same as that shown in Figure 4.Aa. (k) Same as (i), but including $\Delta aceA \Delta dps$ (filled triangles) and $\Delta aceA \Delta rpoS$ cells supplemented with acetate starting $t=0$.

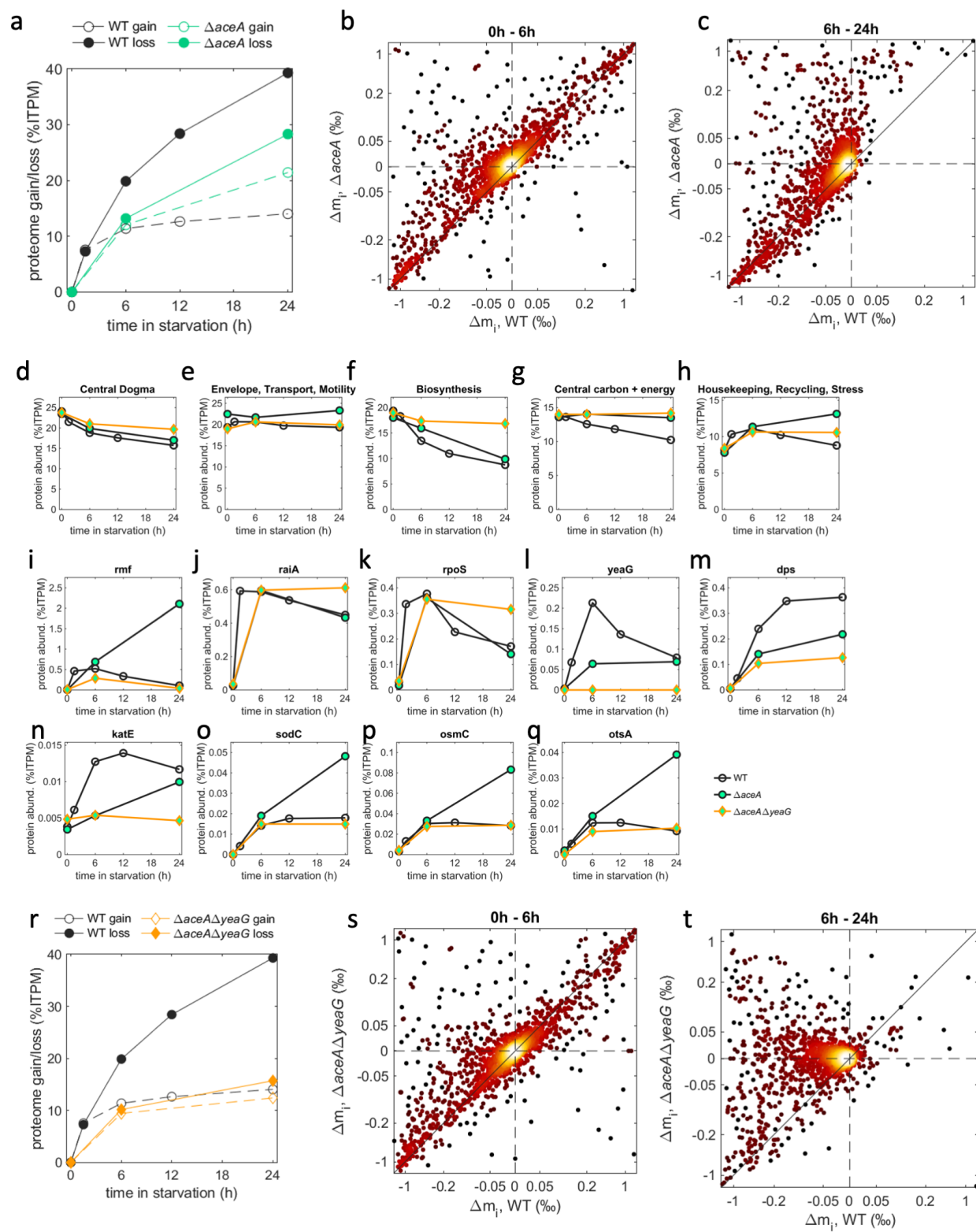


6.2 Analysis of $\Delta aceA$ with acetate supplement proteome during starvation

To see whether the increased viability in energy-supplemented cells is attributable to increased synthesis of stress protein, particularly the protective proteins such as Dps and ribosome hibernation factors, we repeated the proteomic analysis for $\Delta aceA$ cells with acetate supplement after glucose depletion (Figure 6.B). Indeed, many proteins gained in abundance compared to WT cells at the individual level (Figure 6.Ba-c) and for coarse categories (Figure 6.Bd-h, compare open and filled circles). However, a closer look at what proteins increased in gain raises the question about the link to viability. Of the 4 groups that increased for WT cells (Figure 3.Ad), Figure 6.Ae-h (filled vs open circles) show that ribosome hibernation proteins clearly increased compared to WT, while the RpoS regulon level is mixed, with some members increasing, and other member, including Dps, decreasing (Figure 6.Bi-q). Furthermore, substantial increases went to biomass recycling and carbon catabolism (Figure 6.Ag,h), which are not needed in the presence of energy supplement.

Figure 6.B: Proteome remodeling by energy-supplemented $\Delta aceA$ and $\Delta aceA \Delta yeaG$ cells

5 mM of acetate was added to the $\Delta aceA$ cells or $\Delta aceA \Delta yeaG$ cells at the time glucose is depleted, enabling additional energy production but not biosynthesis throughout the first 24h of starvation (Figure 5.D). (a) Protein net gain and loss for $\Delta aceA$ cells compared to WT cells. An increase in net protein gain (open green circles) and a decrease in protein loss (filled green circles) are seen for the $\Delta aceA$ strain, indicating that amino acids from degraded proteins are almost entirely reincorporated into newly synthesized proteins, as expected under the hypothesis that the catabolism is mainly used to generate energy to support protein synthesis, and this catabolism is not needed in the presence of acetate supplement. (b-c) The adaptation of individual proteins in $\Delta aceA$ cells compared to WT, shown in the arc-tangent scale introduced in Figure 4.C. Early adaptation (0 to 6h) is similar between the two strains; the $\Delta aceA$ strain shows additional protein synthesis after 6 hours into starvation. (d-h) Dynamics of the five main protein functional categories for wild type (open black circles; same data as in Figure 3.Bb), $\Delta aceA$ cells (green-filled circles) and $\Delta aceA \Delta yeaG$ cells (orange diamonds). (i-q) Dynamics of ribosome hibernation proteins (Rmf, RaiA) or selected proteins belonging to the RpoS regulon. These proteins display a mixed behavior in $\Delta aceA$ and $\Delta aceA \Delta yeaG$ cells with acetate supplement: Rmf, SodC, OsmC and OtsA reach much higher levels in $\Delta aceA$ cells compared to WT or $\Delta aceA \Delta yeaG$ cells. Instead, levels of YeaG, Dps and KatE are the highest in wild-type. Finally, the levels of RaiA and RpoS reach similar maximum values across strains, with a decrease at 24h in the presence of YeaG. (r-t) Same as panels (a-c) but comparing WT and $\Delta aceA \Delta yeaG$ cells. $\Delta aceA \Delta yeaG$ cells show similar proteome adaptation to early starvation (0 to 6h) but exhibited reduced protein loss between 6h and 24h compared to wild type.



6.3 Effect of energy supplementation on the viability of Δ yeaG cells

To further clarify the role of YeaG-mediated proteolysis, we constructed a Δ aceA Δ yeaG double mutant and characterized this strain in glucose starvation with acetate supplement. This strain exhibited a substantial gain in viability over Δ yeaG (and even WT) cells lacking acetate supplement (filled diamonds, Figure 6.Ai). Total protein exhibited little loss as did Δ yeaG cells (Figure 6.Aj). Proteomic analysis reveals reduced protein gains and losses (Figure 6.Br-t), with the abundances of all stress proteins reduced from those of WT cells (filled diamonds, Figure 6.Ae-h, Figure 6.Bi-q). Thus, YeaG and hence YeaG-fueled proteome remodeling appear unnecessary in extending cell viability in the presence of energy supplement, implicating their functional roles being primarily in helping WT cells to lowering energy demand for cell maintenance in the absence of external energy supplement, e.g., by increasing the ATP conversion efficiency and reducing the futile cycles (Figure 6.Aa). This does not necessarily mean that protective enzymes play no role: *dps*- and *rpoS*-deleted cells lose viability compared to WT cells even in the presence of energy supplement (Figure 6.Ak). Nevertheless, energy supplement reduced the rate of viability drop even for those deletion mutants (Figure 5.Dg,h), indicating that lowering the energy demand for maintenance may be an important function even for these protective enzymes.

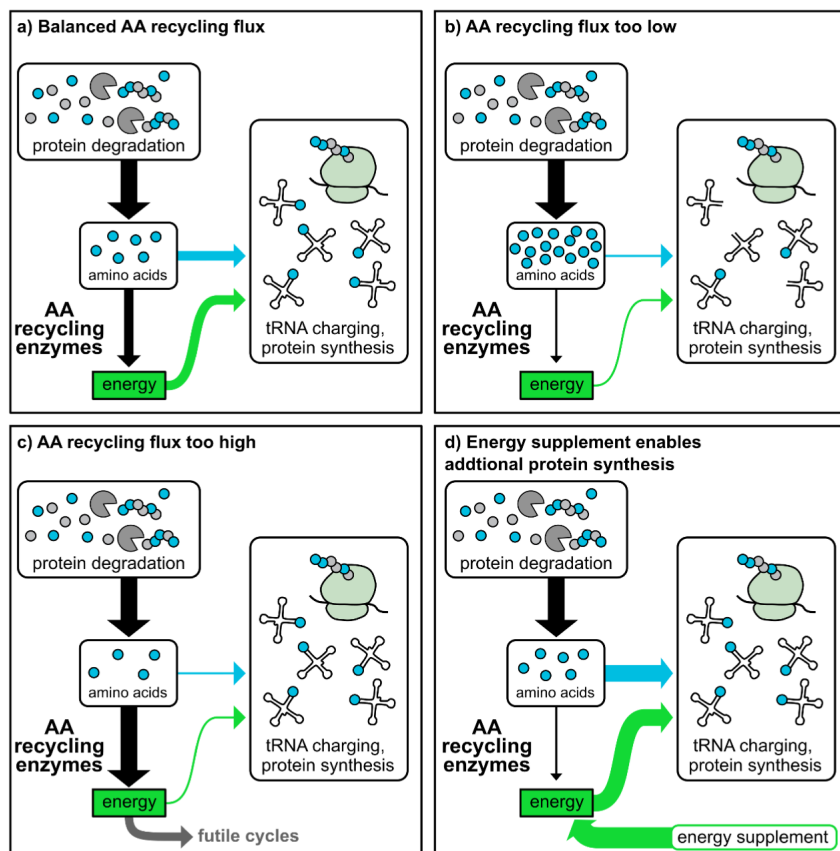


Figure 6.C: Coordination of AA-ATP conversion with protein synthesis

How does the cell set the AA recycling flux, used to catabolize AAs to generate energy, with the remainder used as substrates of protein synthesis? The panels above depict 4 scenarios. (a) depicts the optimal scenario where the two fluxes are just balanced. Setting the AA recycling flux too low would reduce protein synthesis due to energy limitation (panel b) while setting the recycling flux too high would deprive ribosomes of substrates and push energy into fueling futile cycles (panel c). We point out that a natural coordination mechanism exists based on mass action: If the recycling flux is too low, then the resulting energy limitation would first limit tRNA charging, which comprises half of the 4-ATP-per-bond cost of protein synthesis. The slowdown in tRNA charging would in turn result in the rise of the AA pools. This rise would increase the flux of AA recycling towards energy generation, at least by mass action through increase of the substrate pools. The only requirement of this strategy relying on mass action is that the K_M s of the recycling enzymes be larger than those of tRNA synthetases. [Additionally, there could be regulatory mechanisms (transcriptional, translational, or allosteric) to upregulate the expression or activity of the AA recycling enzymes in response to the build-up of AA pools.] In the presence of energy supplement, such as acetate supplement to aceA cells (Figure 5.Da), mass action is sufficient to direct the flux of AA from protein degradation largely to protein synthesis (panel d) as seen in Fig. 5c,d. In this scenario, the residual AA flux going to energy instead of protein synthesis would be given by the ratio of the K_M for protein synthesis over that for energy catabolism.

6.4 Acknowledgements

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Chapter 7: Summary and Outlook

7.1 Contextualizing this work

In my thesis work, we took a deep dive into the physiological response of a K-12 strain of *E. coli*, NCM3722, to carbon starvation. Early in this work it became evident that many of the physiological fundamentals of growing cells that we call upon when studying new features of growing bacteria do not hold in non-growing bacteria. For example, basic features of the *E. coli*'s physiology, such as the rapid degradation of mRNA are altered in the stationary phase: while mRNAs in growing bacteria typically degraded rapidly, on average within 3-5 minutes, studies have shown that many mRNAs can be sequestered in RNase E-mRNA condensates during growth arrest, which slows down their degradation to over 20 minutes^{87,160–162}. Assuming similar mRNA synthesis rates, this change in mRNA degradation would quadruple the effective mRNA pool, which should alter which proteins get translated, especially with fewer active ribosomes due to hibernation. The lack of mRNA dilution via cell division further complicates the picture. Additionally, ribosome hibernation factors are competing with mRNA for ribosomal binding, which further complicates the overall translational capacity and which genes will get translated^{75,135}. These are just two examples of core components of *E. coli*'s physiology which are altered in non-growing cells. Additionally, other physiological components, such as metabolism and morphology, are also altered in non-growing cells which can affect key intracellular molecule concentrations and biochemistry^{11,62,79,163}.

Besides carbon starvation there are myriad stressors that can arrest bacterial growth and varied bacterial responses to growth arrest across organisms^{5,11}. Experimentally, unlike steady-state exponential growth, the stationary phase is a dynamic state, making it difficult to achieve experimental reproducibility and to study isolated cellular processes in a methodical fashion. To make matters more difficult, fundamental tools built for growing cells such as metabolic models need to be reconfigured to be useful in non-growing states¹⁶⁴. Given the complexity and the relative lack of fundamental insights into starved or non-growing bacteria, I believe a focused study of a strain in a specific non-growing state provides value as an entry point. Below, I will summarize some key takeaways from this work and propose questions and considerations for future studies of non-growing bacteria.

7.2 Summary of thesis

7.2.1 Protein degradation dictates protein synthesis in carbon starved cells

In growing cells, protein synthesis is fueled by nutrient uptake while proteolysis provides auxiliary services such as quality control and alternative means of regulation¹⁵⁰. During carbon starvation, however, as we define in Chapters 2 and 5, proteolysis becomes the primary means by which a starving cell utilizes its own biomass as a reserve resource for protein synthesis and other energy needs. Thus, proteolysis fundamentally occupies a position “upstream” of protein synthesis in starving cells. In Chapters 3 and 5 we show that, due to the huge cost of protein synthesis, ~2 proteins are degraded for each protein synthesized (Figure 5.Ac) or 5-6 proteins are degraded for each protein gained (Figure 5.Ae).

Starving cells must resort to global proteolysis to synthesize even a moderate amount (~10% ITPM) of stress proteins to sustain survival (Figure 4.Ab). The large cost of protein synthesis provides a rationale for why the alternative demand-driven strategy is not selected: if there is a demand from transcription to make 20% ITPM of certain proteins, then a cell would need to turnover over more than 100% of its proteome to meet this demand (and would die along the way). Instead, the supply-driven strategy provides cells with a set amount of resources to prepare for long-term survival. *E. coli* manages its proteome remodeling dynamics very carefully, via a supply-driven strategy of resource allocation (Figure 5.Cb) implemented by YeaG.

7.2.2 YeaG facilitates protein degradation and synthesis

In Chapters 4 and 5 we identify YeaG, a highly synthesized protein in carbon starvation under the transcriptional control of RpoS, as the facilitator of a wave of widespread protein degradation and, consequently, an enabler of protein synthesis. YeaG is synthesized from negligible levels during growth to ~0.2% ITPM, or ~10,000 copies per cell in the first hours of carbon starvation (Figure 4.D), leading to an increase in degradation flux 3-5x over the basal degradation rate (Figure 5.A).

This work adds to the limited but growing literature on YeaG and its role in non-growing bacteria^{142-144,151,165}. Previous work has attempted to address YeaG's mechanism of action, focusing on the putative kinase domain, and its role in the bacterial response to nitrogen starvation. By elucidating YeaG's role in facilitating protein degradation this work could help focus future mechanistic studies and contextualize its effects in other starvation

conditions. Regarding mechanism, we present evidence that both the eSTK and AAA+ domains are important for protein degradation and cell viability (Figure 4.B). YeaG appears to operate in conjunction with other gene on its operon, YeaH, and to act through ClpP or ClpXP (Figure 4.A). Together, these results suggest YeaG may be an unfoldase that boosts ClpP degradation activity. Future molecular characterization, including *in vitro* and structural studies, will be exciting and impactful given the rarity of elucidating a novel protein degradation element in *E. coli*, especially if it can be harnessed as a molecular research or engineering tool.

7.2.3 YeaG-enabled turnover improves cellular energy status and survival

Proteins are degraded to provide resources for new protein synthesis at a cost of ~2 proteins degraded for every protein synthesized (Figure 5.Ae), leading to a remodeling of the proteome and reduction in total protein mass of ~25% in the first 24 hours of starvation. This costly process is valuable in that it allows cells to maintain viability for up to 4 days (Figure 2.Bb). Our results show that YeaG-mediated remodeling is unnecessary when exogenous energy is supplied (Figure 6.Ai). This suggests that, in wild-type cells, the synthesized proteins either reduce futile energy expenditure, enhance energy production via increased amino acid recycling, or achieve both. Additionally, the act of degrading growth-associated proteins itself may decrease energy demand. There are, however, some non-energy related functions of certain proteins in the RpoS-regulon such as Dps, which are required to a certain degree even with energy supplement to maintain viability (Figure 6.Ak).

Overall, this work introduces an integrated approach that links the allocation of limited resources to global gene expression and ultimately population survival. It provides a valuable entry point into the complex landscape of stationary phase physiology, offering numerous opportunities for further research and potential practical applications, some of which I will introduce below.

7.3 Outlook

I will reflect on a few of the future scientific questions that could benefit from the approaches and insights presented in my thesis. I have conducted preliminary studies for related to some of these questions, which I will summarize with the understanding that further research is required to gain definitive insights.

7.3.1 Inducing gene expression in stationary phase

In addition to the gene expression programs bacteria have evolved to survive carbon starvation, it is important for cells to have the capacity to synthesize additional proteins during starvation in response to the introduction of novel carbon sources or additional stressors beyond carbon starvation^{8,21,166}. Additionally, induced protein expression out of the growth phase may be important for engineered functions^{167–169}. Natively, to utilize a novel carbon source, a cell initially needs to synthesize only a small amount of the relevant transporters and catabolic enzymes. Once these proteins are produced, the novel carbon source can be catabolized to fuel further protein synthesis, ultimately supporting cell growth and division^{44,90,99}. However, to further study cellular constraints and the supply-driven model of protein turnover in stationary phase and to enable applications where significant

protein synthesis is needed, we began work towards constructing in which gene expression can be titrated to appreciable levels in starving cells, say, to ~5% ITPM, which is half of the 10% ITPM protein gained in WT cells.

We constructed a strain in which synthesis of the reporter protein LacZ^{170,171} could be induced throughout the first 24 hours of carbon starvation (Figure B1a), replicating a ‘constant activity stationary phase (CASP)’ phenotype presented previously¹⁷² (for preliminary data and more details, see Appendix B1). Expression attained from inducing the expression of a single-copy LacZ on the chromosome had minimal effect on viability (Figure B2a). Attempts to induce stronger gene expression using a high-copy plasmid resulted in limited functional LacZ expression (Figure B1b) but a strong effect on cell viability, with ~50% cell death in 24 hours (Figure B2b). We speculate that this lethality may arise from the energy cost of mRNA synthesis itself, given the high ATP demand (6 ATP per codon¹⁷³). Alternatively, LacZ mRNAs may persist within condensates¹⁶², continuously engaging ribosomes without completing translation cycles, thereby futilely consuming cellular resources. In future work, the cost to viability of mRNA synthesis alone can be tested with the same plasmid-based LacZ overexpression system but with a low affinity RBS such that translation will not be initiated.

Together, these preliminary findings highlight the need for caution when manipulating gene expression in carbon starvation conditions and only begin to explore the physiological constraints on gene circuit design in stationary phase broadly—a challenging but exciting goal for synthetic biology^{174–176}.

7.3.2 Starvation of other essential nutrients

Starvation of other key nutrients can be studied utilizing the approach presented in this work. The depletion of each distinct nutrients essential for growth presents a unique challenge to cell survival, as each nutrient plays a different critical role in cell physiology^{5,106,177}. Additionally, physiological responses to starvation will be tailored to the ecological niche within which the bacteria have evolved. For example, nitrogen appears to not be limiting in the mammalian gut⁴⁰ but in the ocean nitrogen is considered the most limiting essential nutrient¹⁷⁸.

Nitrogen is a fundamental component of amino acids, and previous work has measured the turnover of proteins in nitrogen starved *E. coli*⁶⁷. In preliminary work, I found that nitrogen starvation poses a challenge for WT cells and that YeaG is important for survival under nitrogen runout conditions (Appendix C). Although the strains, media, and specifics of the viability curves differed, this result builds on a previous study that demonstrated a viability defect in a $\Delta yeaG$ *E. coli* strain¹⁴². In fact, in my experimental conditions, both WT and $\Delta yeaG$ cells died faster in nitrogen starvation than in carbon starvation (Figure C1). These initial data suggest YeaG-enabled proteome degradation may be important in a range of non-growing conditions, an exciting prospect for future work. Additionally, nitrogen starvation presents a particularly interesting condition for industrial bioproduction of molecules which I discuss in section 7.3.4.

7.3.3 Starvation and YeaG in other bacteria

YeaG (alongside *yeaH*) is also easily found by DNA sequence homology in pathogenic bacteria such as *Vibrio cholerae* (homologous gene: VAB027_RS12740) and *Pseudomonas* (homologous *YeaH* gene: PA50071_RS02945, unannotated genes upstream and downstream). The central role of protein degradation in stationary-phase metabolism presents a case for antibiotic development targeting this process. In fact there have been previous efforts to develop drugs that inhibit protein degradation, including targeting ClpP^{179–181}. Recognizing the importance of protein degradation for survival in stationary phase bacteria could renew interest in such approaches.

7.3.4 Relevance for metabolic engineering and industrial biotechnology

Industrial biotechnology involves the production of molecules using cellular metabolism or *in vitro* enzymatic pathways¹⁸². When using cells for bioproduction, it is important to grow cells to high densities to maximize production per unit volume—a process that is both costly and time-consuming. To mitigate this cost, a high density cells would ideally be maintained for a long period of time in non-growing but metabolically active state, producing a molecule of interest via a stable, engineered metabolic pathway¹⁸³. However, productivity often declines rapidly, often in a matter of days, once cells reach stationary phase due to nutrient limitation or other stressors^{183–185}. Extending the productive phase even by a few days would be highly beneficial and could open new markets bioproduction of lower-priced molecules. However, despite extensive research in both academic and industrial settings to identify and mitigate the causes of declining culture productivity,

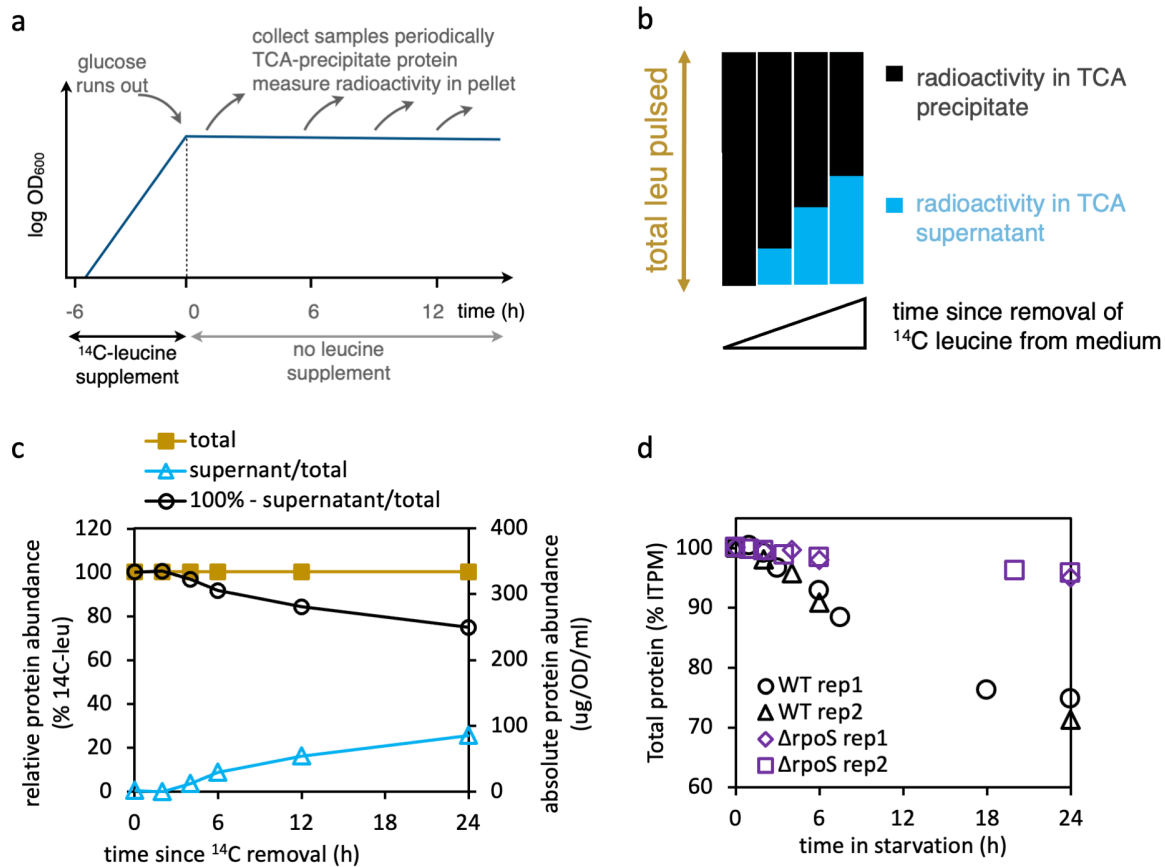
productivity loss remains a significant challenge^{185–187}. In this context, YeaG-mediated degradation of proteins, including metabolic enzymes (Figure 3.A), could be a potential cause of productivity loss in engineered strains, where it might degrade key enzymes involved in the biosynthesis of desired products. Additionally, our study, along with previous research, suggests that metabolically active cells tend to die faster in stationary phase^{62,142}, which presents a challenge to engineer strains that are productive in a non-growing state. Energy supplementation in a $\Delta yeaG$ strain may offer a solution by preserving useful proteins while providing sufficient energy for continued metabolic activity, enabling the cells to function effectively as a biofactory. Energy supplementation could be accomplished by feeding acetate to $\Delta aceA \Delta yeaG$ cells or by applying the same logic using another carbon source that cannot be metabolized for growth, depending on the particulars of the strain and conditions.

Appendix A: Radiolabeling Method Development

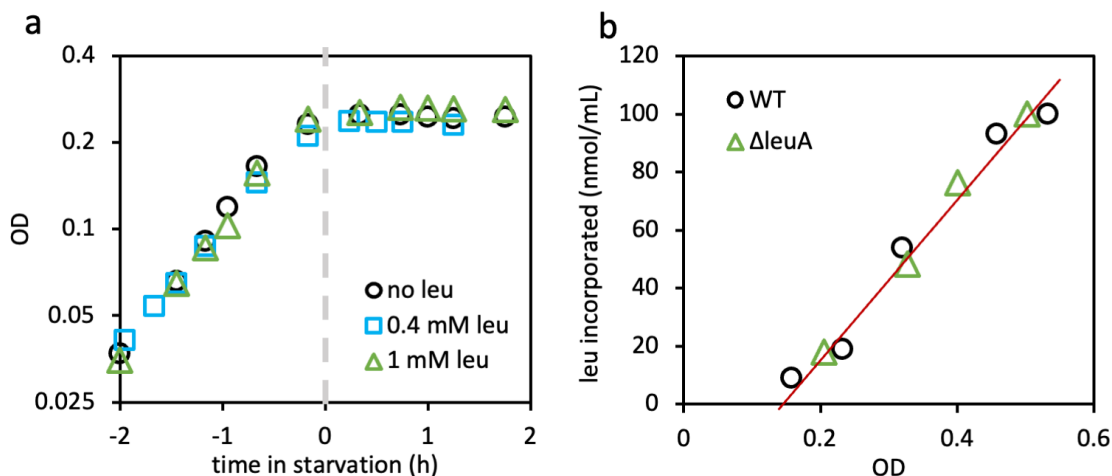
Various radiolabeling methods, originally developed in the 1950s and later utilized by microbial physiologists through the 1980s^{66,67,71,188}, were revived to precisely measure total protein levels, as well as protein synthesis and degradation. The methods are described in the figures below.

A.1 Total protein abundance measurement

For sensitivity and reliability, we use a radio-labeling approach to quantify the amount of proteins in a culture. (a) Illustration of the experimental procedure. To label intracellular protein, cells were grown in the presence of ^{14}C -leucine until glucose runs out ($t=0$) at OD » 0.4 (Fig. 1a). (Leucine supplement does not affect exponential growth nor viability during glucose starvation, see Appendix A2). Unincorporated leucine is immediately removed from the medium by washing with media lacking glucose. Washed cells were resuspended in fresh medium without glucose (and no leucine), but additionally supplemented with 0.86 mM sodium acetate. The acetate supplement was included to reconstitute the medium at the time of glucose depletion, which contained 0.86 mM acetate due to overflow metabolism during exponential growth on glucose (about 2mM acetate/OD)⁷⁶; no other metabolites were detected in the medium during growth^{93, 189}. Aliquots of the resuspended culture were sampled at regular time intervals and were precipitated with trichloroacetic acid (TCA). (b) The direct method to measure protein abundance would be to measure the radioactivity in the precipitate (black bars). However, this approach gives rise to variable results due to, e.g., loss of the precipitated materials during subsequent acetone-washes (see Chapter 2 Methods). Instead, we measure the radioactivity in the supernatant (blue bars). As the total radioactivity of the culture (brown arrow) cannot change, a decrease in the radioactivity from the TCA precipitate is coupled to an increase in the radioactivity of the supernatant. Thus, by monitoring the (much more accessible) radioactivity in the supernatant (Chapter 4 Methods), radioactivity in the precipitate (and hence protein abundance) can be quantified. This problem with TCA precipitate is not limited to radio-labeling, but affects common approaches to measuring protein abundances, e.g., the Bradford assay¹⁹⁰. It is for this reason that we use the radiolabeling assay instead of the common assays to quantify protein abundances throughout this work. (c) WT culture was grown in glucose + ^{14}C -leucine and washed at $t=0$ as described in panel (a). Samples taken during glucose starvation were analyzed. Brown squares represent total radioactivity measured in the sampled culture, taken as 100%, and blue triangles represent percent of total radioactivity in the supernatant. Black circles are the differences between brown and blue, which we take to be radioactivity in the TCA precipitate, reflecting the total protein amount remaining in the culture. The left vertical axis is the fraction of radioactivity in each component of the culture. The right axis represents the same data, but on the scale of absolute protein abundance, taking the measured protein abundance in exponential growth⁷⁶ (333 $\mu\text{g}/\text{OD}/\text{ml}$) as the protein abundance at $t=0$. (d) The results obtained in this way are highly repeatable, as shown by independent biological replicates for WT and $\Delta rpoS$ strains (black and purple symbols, respectively) during the first 24h of glucose starvation.



A.2 Validation of leucine-labelling to monitor protein abundance

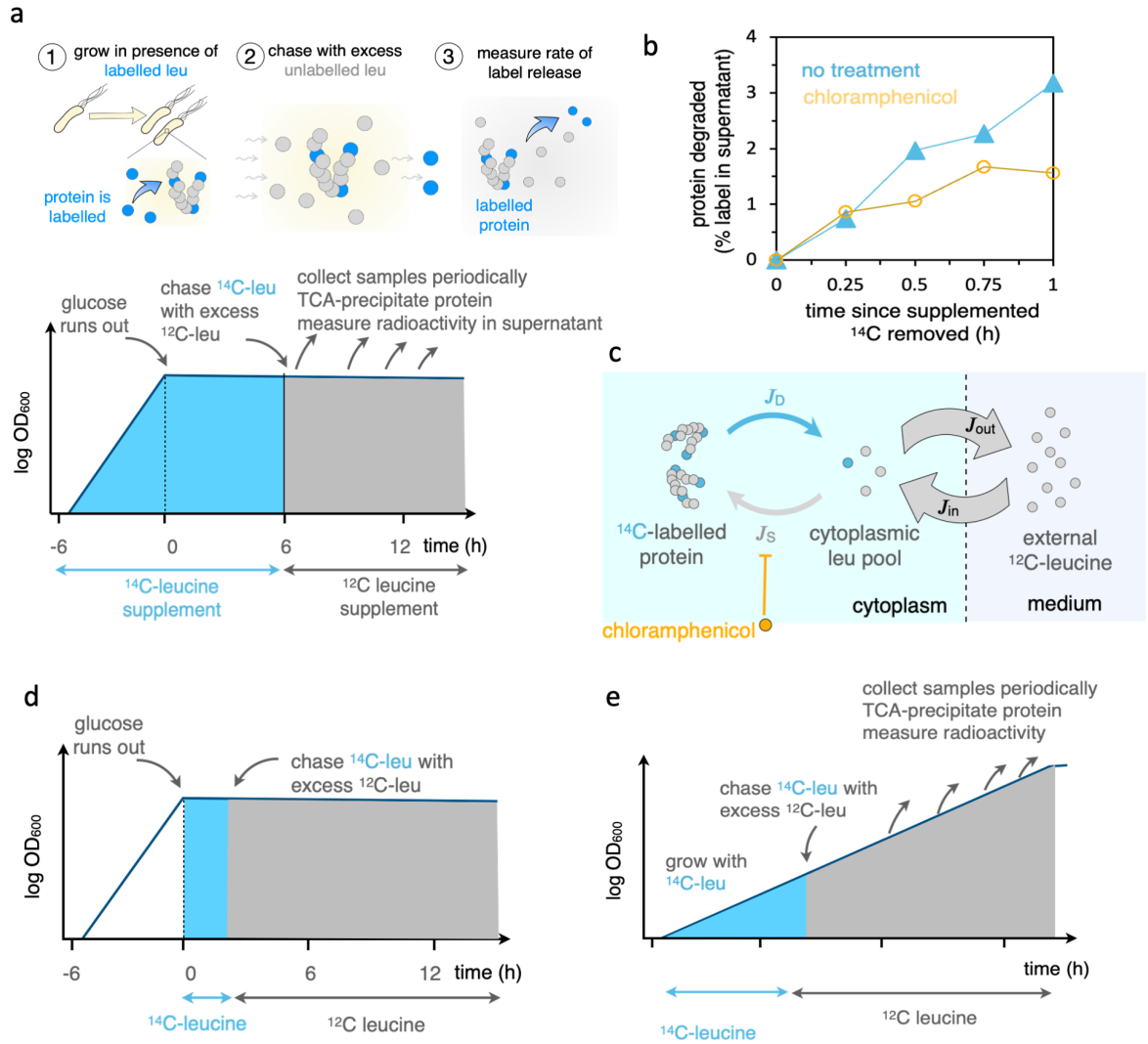


a) The presence of external leucine has no detectable effect on growth rate and yield of *E. coli*. This is expected as *E. coli* cannot be grown on leucine as the sole carbon source and lacks leucine degradation pathways (Figure 2.D: Catabolism of amino acids for energy generation), making it an ideal choice for radiolabeling experiments. (b) Leucine incorporation into proteins (TCA precipitable fraction, Appendix A1) for WT cells (circles) and leucine auxotroph, $\Delta leuA$ cells (triangles), plotted at different culture OD₆₀₀ during exponential growth on glucose supplemented with 400 μ M leucine. Comparable leucine incorporation by WT and $\Delta leuA$ cells suggests that when supplemented with leucine, *de novo* leucine biosynthesis is negligible for WT cells. Thus, the relationship between radioactivity and leucine content (specific radioactivity of leucine) in proteins is the same as that for externally provided leucine, as assumed in the analysis of Appendix A1c. The straight line is a two-parameter linear fit of the data. The x-intercept of the fit (~ 0.15 OD) corresponds to the loss in biomass during centrifugation procedures (see Methods). The slope, 265 nmol/OD/mL is equivalent to 8.8 % of protein content (3 μ mol/OD/mL based on 330 μ g protein/OD/mL [Basan15]). This matches closely the leucine frequency of 8.7% listed in Table 2.3. This agreement further reinforces the lack of leucine catabolism. (c) Wild type cultures were grown in the presence of 2 mM glucose and 1 mM leucine (yellow triangles) until glucose depletion. CFU of the glucose-starved culture (containing ample leucine) was followed for several days subsequently and compared to that of the natural glucose runout without leucine (Figure 2.Bb). The presence of leucine has a negligible effect on viability. (d) Compare viability of WT runout with WT wash + 0.86 mM acetate.

A.3 Degradation extent and rate measurement

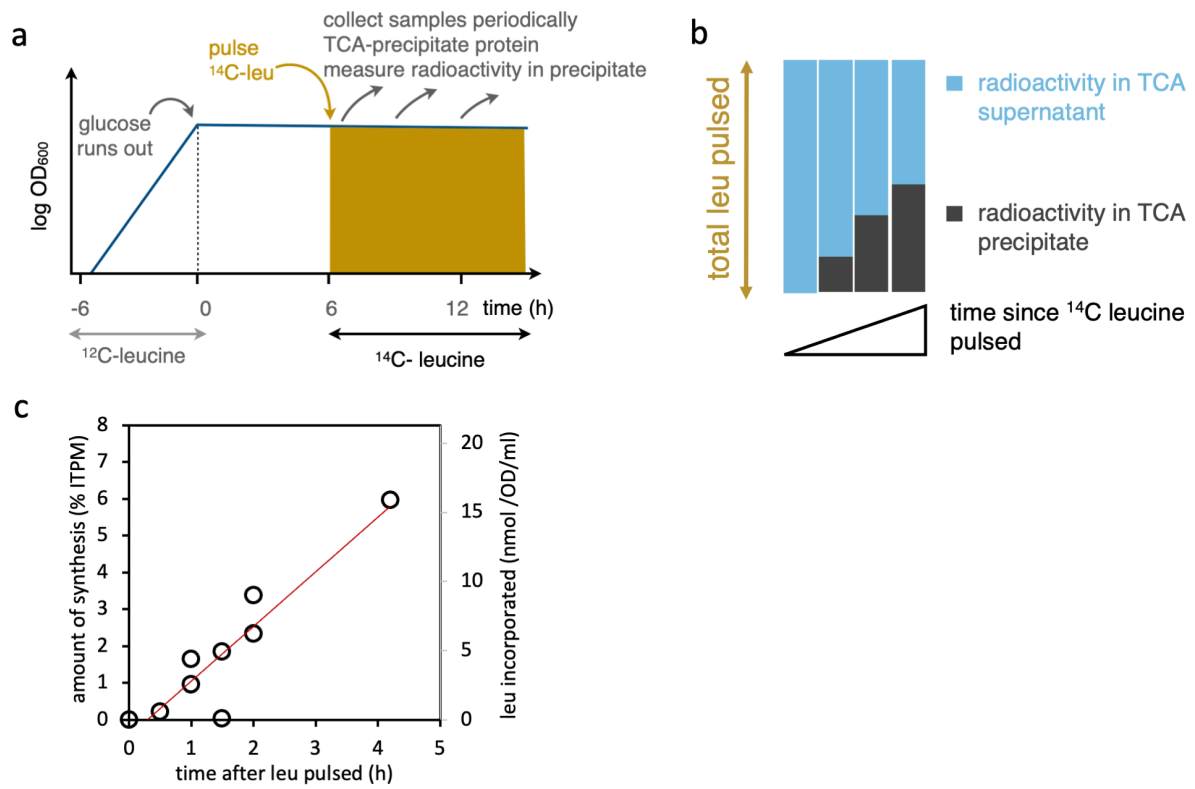
(a) Illustration of the experimental procedure. To label intracellular protein, exponentially growing cells were inoculated into the same glucose medium as described in Figure 2.Aa, except for the supplement of 100 μ M ^{14} C-leucine. The cells grew for ~ 4 doublings until glucose ran out at $OD_{600} \gg 0.4$ ($t=0$). At the desired time for which the extent of protein degradation was to be measured (for example, at 6h into starvation as illustrated), unincorporated leucine was removed from the culture by washing with medium lacking glucose and leucine. Cells were resuspended in fresh medium without glucose but containing excess (1 mM) ^{12} C-leucine to initiate the chase of the radiolabel. Aliquots of the culture were sampled at regular time intervals and precipitated with trichloroacetic acid (TCA). Radioactivity in the TCA supernatant was measured to monitor the release of ^{14} C-leucine from protein degradation. The data Figure 4.Ac, d, and Figure 4.B were taken this way, with the wash of ^{14} C-leucine and the chase of ^{12} C-leucine occurring at the time of glucose runout ($t=0$). (b) For the measurement of “instantaneous” protein degradation flux, the amount of radioactivity in the TCA supernatant was measured at short (15 min) intervals after chase. The results, plotted as blue triangles, are expressed as percentages of the total radioactivity in the culture at the moment of chase. This represents a lower bound on the fraction of proteins degraded since the time of chase. The line fitted to the blue triangles indicates a degradation rate of $\sim 3.3\%$ proteins/h. The gold circles represent the same measurements repeated by adding chloramphenicol (50 μ g/ml) at the moment of chase (see below). The protein degradation flux values shown in Figure 5.Aa are obtained by further dividing the instantaneous rate as measured in panel a and b by the instantaneous protein abundances (Figure 4.Aa). (c) The expected dynamics of leucine exchange after the chase. Immediately after chasing the label with excess of non-labelled leucine, all label (blue circles) is present within proteins. Over time, the label is released into the cytoplasmic pool at a rate that depends on the protein degradation (J_D) and synthesis (J_S) fluxes. Furthermore, the cytoplasmic leucine pool rapidly equilibrates with the non-labelled excess leucine in the medium (gray circles). Under the assumption that the fluxes J_{in} and J_{out} (occurring at timescales of seconds) are much larger than J_D and J_S (occurring at timescales of hours), reincorporation of released label would be negligible. This assumption is tested by adding chloramphenicol at the moment of chase to inhibit J_S . If reincorporation of label into synthesized proteins is appreciable, then chloramphenicol treatment would result in an increased release of label. This is ruled out by data shown in panel b (compare yellow circles and blue triangles), validating that reincorporation of label is negligible. Thus, the rate of label accumulation measured in panel b can be taken as the protein degradation rate at the time of measurement. (d) Unlabeled cultures were grown in glucose and ^{14} C leucine was added at the moment of glucose depletion. Thus, only proteins synthesized after $t=0$ are labelled. After 2 hours in starvation, the label is chased with ^{12} C leucine allowing the measurement of the degradation rate of the proteins synthesized in starvation. This procedure was also repeated for adding ^{14} C leucine 2h after glucose depletion and chasing with ^{12} C leucine 2h later to obtain the degradation rate of proteins synthesized during $t=2-4$ h after starvation. (e) Exponentially growing cultures in the presence of 50 mM ^{14} C leucine were shifted to an otherwise identical

medium but containing 1 mM ^{12}C leucine instead of the label, and degradation was monitored while growth continued.



A.4 Synthesis flux measurement

(a) Illustration of the measurement procedure. Cells are grown exponentially until glucose runs out at $OD \approx 0.4$ ($t=0$) as shown in Figure 2.Aa. At the desired time of measurement, 6h in this example, the culture is pulsed with a small amount of ^{14}C -leucine. The cultures are then sampled at regular time intervals and precipitated with TCA. (b) All of the leucine pulsed in at 6h (brown arrow) would be contained in the TCA supernatant initially. As proteins are synthesized, the ^{14}C level in the supernatant (blue bars) would decrease while that in the precipitate would increase (black bars). The amount of leucine incorporated into proteins can thus be obtained by monitoring the radioactivity in the supernatant; the absolute abundance obtained from the known relation between radioactivity and leucine concentration, and the leucine composition of the proteome. (c) Wild type cells are grown as described in panel a and ^{14}C leucine is pulsed at 6h of starvation. Samples are collected at short time intervals and precipitated with TCA. The amount of leucine incorporated into proteins, shown on the right axis, is taken to be the total radioactivity pulsed in minus the radioactivity measured in the supernatant, at ~ 1500 CPM/nmol. From the known leucine content of cells (265 nmol leucine/OD/mL, or 106 nmol for 1 ml of culture at $OD \approx 0.4$, Appendix A2 the leucine incorporation rate can be converted to a percentage of the initial total protein mass (left axis): Amount of protein synthesized (as % ITPM) at time t = nmol of leucine incorporated at time t per OD·ml of the initial culture $\div$ 106 nmol/iOD/ml $\times 100$. The flux of protein synthesis is the slope of the linear-fit of the data. The data in Figure 5.Ab are obtained in this way for cultures with ^{14}C -leucine pulsed at various time after starvation, with the linear fit over data taken for a period of 2 hours at the interval of 0.5 hours for measurements within 12 hours of starvation, and over a period of 4 hours at the interval of 1 hour for measurements made after 12h of starvation.



Appendix B: Inducing gene expression and effects on viability

B.1 Inducing gene expression, preliminary work

The dominant sigma factor in growing *E. coli* cells, RpoD (σ^{70}) enables the transcription of the majority of *E. coli* promoters and is the most abundant and active sigma factor during growth^{44,84,191,192}. During starvation, there are various mechanisms leading to diminished RpoD output, including competition with other sigma factors and the shutting down of RpoD activity via 6S RNA and the anti-sigma factor *rsd*^{87,193–197}. These mechanisms could explain why we observe a reduction in RpoD activity reflected in our proteomics data. Net gain of catabolic genes (which are under RpoD control) ceases after 6 hours of starvation (Figure 3.Ad) and gain (and by deduction synthesis) of an exemplary periplasmic catabolic protein RbsB, which should be protected from cytosolic YeaG-enabled degradation, ceases by 12 hours (Figure 3.C). It was not wholly surprising then that induction of β -Galactosidase (LacZ), a CRP-regulated catabolic gene frequently used as a reporter for gene expression^{170,198}, became negligible by 20 hours into starvation when expressed from its native promoter (black triangles, Figure B1a).

We constructed a strain in which LacZ was induced from an orthogonal sigma factor, RpoN (σ^{54}). We harnessed the Pu promoter from the TOL plasmid from *Pseudomonas putida*, which is controlled by a constitutively expressed XylR activator whose activity is controlled by an exogenously supplied molecule 3MBA^{199,200} (see 2.6.3 Strain Construction, Table 2.4 and B.3 Methods). Using this strain, we were able to consistently induce gene expression throughout the first 24 hours of starvation, (green circles, Figure B1a), with the amount of

functional LacZ obtained being 10x more than that of the LacZ induced from its native promoter (black triangles). An attempt to increase LacZ expression further by harboring Pu-LacZ on a multicopy plasmid resulted in ~10-fold lower levels of LacZ induction with precise results varying from run to run (different symbols in Figure B1b), when induced 6 hours into starvation, where the protein synthesis capacity of WT cells is high (Figure 5.Ab). Although these are preliminary results, they highlight potential challenges when inducing gene expression during carbon starvation, with plasmid systems potentially presenting extra challenges.

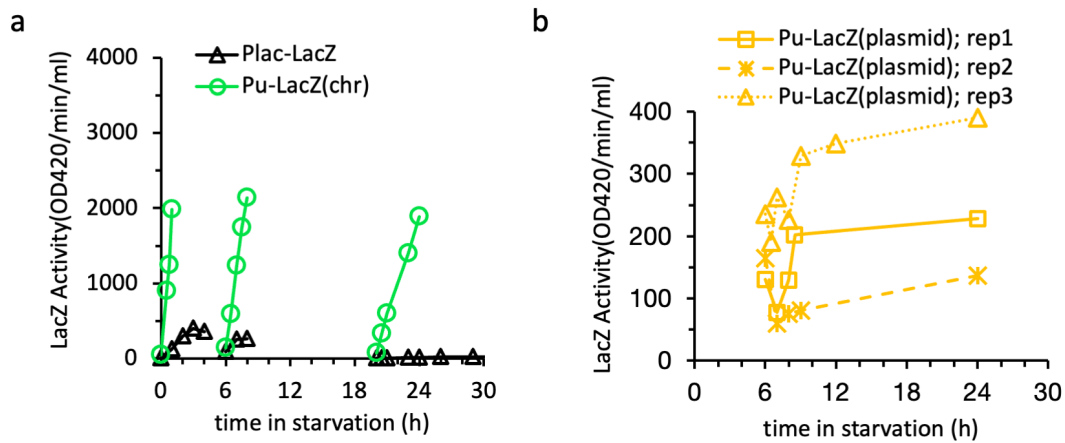


Figure B1: LacZ induction

(a) Chromosomal LacZ was induced at 0, 6, and 20 hours into glucose starvation under the control of different promoters (see B.3 Methods). Plac-LacZ (RpoD promoter) in its native position on the chromosome in WT cells was induced by 1mM IPTG (black triangles). Pu-LacZ (RpoN promoter), driven by a constitutively expressed XylR activator, was induced by 3MBA (green circles). Rates of induction, taken to be proportional to the amount of functional LacZ enzyme, depend on details of promoter strength and induction system. Note that these data are not normalized to culture OD₆₀₀ (iOD nor OD at time of sample collection) but growth arrested at ~0.375-0.425 for all cultures and does not greatly affect the analysis. (b) Pu-LacZ on a multi-copy plasmid (strain HE1010, with pZE12-Pu-LacZ plasmid which has a ColE1 ori, typically 70-100 copies in growing cells²⁰¹) was induced, yielding diminished production of functional LacZ (yellow symbols, each symbol corresponding to one distinct biological replicate). For all experiments, data point represents LacZ levels as quantified LacZ activity (see B.3 Methods).

B.2 Effects of inducing gene expression on viability, preliminary work

We compared the effects of inducing Pu-LacZ expression from the chromosome to induction from a high-copy plasmid. Chromosomal induction resulted in minimal viability defect whereas induction of LacZ on a multi-copy plasmid resulted in significant viability loss; death of 50% of the culture in 24 hours. The rapid loss of viability of the Pu-LacZ (plasmid) strain is not directly explained by functional LacZ production (Figure B1b), nor by the constitutive expression of XylR or 3MBA since both conditions were present in the Pu-LacZ(chr) strain. We hypothesize that there might be excess mRNA production draining energy and perturbing normal translational processes in the plasmid strain.

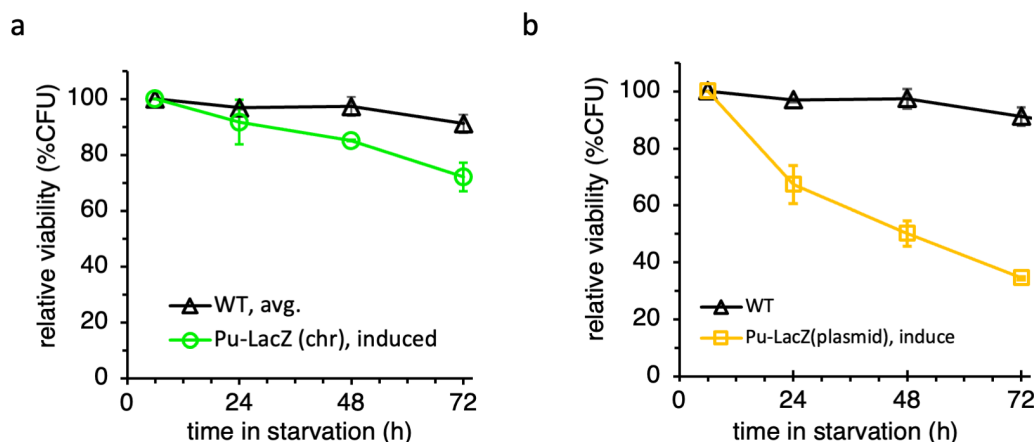


Figure B2: Effect of inducing LacZ on viability

(a) Viability of strain NQ537 a chromosomal LacZ strain induced from Pu promoter (green circles). (b) Viability of strain HE1010 with LacZ in a multi-copy plasmid induced from the Pu promoter (yellow squares). WT viability curve (uninduced) is included for reference (black triangles, same data as Figure 2.Ab).

B.3 Methods

LacZ induction was performed at various times during glucose starvation in three strains (WT, NQ537, HE1010; see Section 2.6.3, Strain Construction, and Table 2.4) using two different gene expression systems (Plac-LacZ and Pu-LacZ). In WT cultures, LacZ expression, driven by its native Plac promoter and located at its native chromosomal locus in the lac operon, was induced by adding 1 mM IPTG to cultures. In the strains with Pu-LacZ (NQ527 with chromosomal Pu-LacZ; HE1010 with plasmid-based Pu-LacZ), the activator of the Pu promoter, XylR, was expressed from a constitutive RpoD-controlled promoter, P_{Lac} . To activate the Pu promoter via XylR, 800 μ M 3MBA was added^{199,202}.

To measure LacZ levels, 1.5ml samples of cell culture were collected and immediately frozen on dry ice before storage at -20°C. Samples were thawed and LacZ activity was quantified using the standard biochemical Miller assay¹⁷⁰, in which LacZ activity is proportional to its protein level. Typically, LacZ levels are reported in Miller Units, which requires fitting a line through multiple LacZ activity measurements across a steady state growth curve. But since these samples are taken out of steady state growth, we report LacZ activity (OD420/time/sample volume) in the culture over time.

Appendix C: Nitrogen runout

C.1 Survival during nitrogen starvation

We briefly explored the survival of nitrogen-starved WT and $\Delta yeaG$ cells using the same method as the carbon runout experiments (see Section 2.6.2, Cell Growth and Starvation), but with a limiting amount of ammonium (the sole Nitrogen source) added to the growth medium instead of limiting glucose. 1.4mM ammonium chloride and 10mM glucose was included in the experimental culture, leading to abrupt growth arrest due to nitrogen depletion at $\sim 0.4OD$ (Figure C1a). The survival of nitrogen-starved WT and $\Delta yeaG$ strains was lower compared to carbon starvation conditions (Figure C1b).

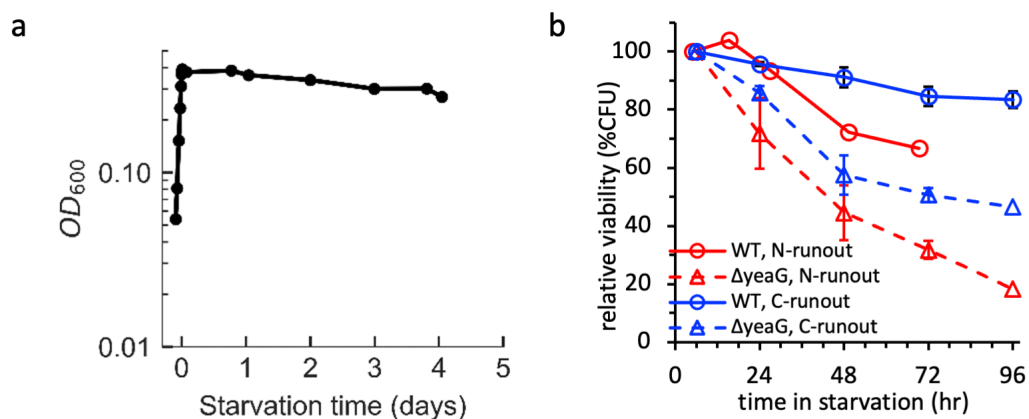


Figure C1: Effect on nitrogen starvation on viability

(a) Cell density (OD_{600}) of a WT culture with 1.4mM ammonium chloride and 10mM glucose leading to growth arrest at $\sim 0.4OD_{600}$. (b) Viability of nitrogen-starved WT (red circles, solid line) and $\Delta yeaG$ cells (red triangles, dashed line). WT (blue circles, solid line) and $\Delta yeaG$ (blue triangles, dashed line) carbon starvation viability curves are included for reference (same data as Figure 2.Ab and Figure 4.Ab).

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