Stress-induced metabolite release creates an ecological and evolutionary opportunity for restoring growth regulation

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Abstract

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Nutrient sensing is a fundamental component of eukaryote survival. In order to convert environmental stimuli to a proper physiological response, many systems like the budding yeast *Saccharomyces cerevisiae*, rely on tight regulation by complexes such as TOR. Yeast, when starved for natural nutrients such as glucose, repress TOR signaling and undergo a quiescence phenotype. However, when yeasts are made auxotrophic for certain compounds, like the amino acid lysine, this 'unnatural' nutrient limitation is not properly regulated: cells continue to waste glucose and rapidly decrease in viability. This differential response to various types of nutrient limitation ('natural' vs 'unnatural') suggest differing evolutionary pressures to properly maintain adequate nutrient sensing. To study how cells could adapt to an 'unnatural' nutrient limitation, I evolved a lysine auxotroph of *S. cerevisiae* under lysine limitation. As expected, many strains had gained mutations that increased the affinity for lysine. However, I found that a significant percentage of my clones had become auxotrophic for metabolites (organosulfur and glutamine) that had not been supplied exogenously. The nature of these autotrophies was suggestive the

reemergence of TOR-regulated metabolite sensing, as these classes of metabolites have previously been shown to interact with TOR and other similar effectors.

To understand how this was possible for the nascent organosulfur auxotrophs, I first used a combination of phenotypic assays and metabolomics to determine that live lysine-requiring cells when limited for lysine release glutathione in a stress-dependent manner. New organosulfur auxotrophs, when supported by this emergent pool of glutathione, were able to rise to high frequencies through a negative frequency-dependent fitness benefit compared to organosulfur prototrophs. This adaptive benefit appears to be due to enhanced survival under lysine limitation, mediated through the regaining of TOR signaling, as the removal of a downstream process, autophagy, significantly diminishes the survival phenotype.

This study demonstrates the cells can generate their own ecological pressures which may in turn drive the emergence of novel metabolic strategies, a finding with consequences both at the cellular and community level.

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ACKNOWLEDGEMENTS

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DEDICATION

To Mom, Dad, and Lexie.

You make me feel like I can do anything.

Chapter 1. NUTRIENT SENSING AND REGULATION: A FUNDAMENTAL PROCESS, BUT ONE THAT IS EASILY CIRCUMVENTED

1.1 NUTRIENT INFORMATION MUST BE SENSED AND RELAYED

For an organism competing in any environment, it is essential to translate external stimuli to a proper physiological response. All known life requires basic building blocks (herein referred to as 'natural metabolites') such as carbon, phosphorus, nitrogen, and sulfur. Failure to properly regulate responses to nutrient signals can result in unchecked growth (which is often medically associated with tumor development[1]). Prolonged existence in this state can lead to resource wasting[2,3] and rapid cell death[4]. Given the importance of these classes of metabolites, it is unsurprising that there is a strong evolutionary pressure to tightly regulate how cells sense, process, and respond to nutrient signals in the environment. Many common regulators of nutrient sensing are at least moderately conserved among eukaryotes[5]. In order to mechanistically understand these regulators and the processes they control, many laboratory studies employ 'omics' approaches to well-studied systems in a controlled environment of limitation for a single nutrient. Readouts in the form of transcriptomics [6], metabolomics[7,8], and proteomics[9] can be used to assess the physiological response of a model organism to nutrient limitation and define what is considered a 'normal' or 'proper' physiological response.

1.2 'PROPER' PHYSIOLOGICAL RESPONSES TO NUTRIENT DEPRIVATION IN YEAST

In the model laboratory system *Saccharomyces cerevisiae*, extensive work has been done to understand how this organism responds to limitation for nutrients, both for the sake of academic insight and also with the hope that the evolutionary conservation between humans and

yeast[10] can lead to novel understanding of disease. To do this, many studies have employed the chemostat, a device that can maintain cells at a constant physiological state of limitation for a single nutrient through controlled delivery at a fixed rate [11]. Transcriptomic analysis revealed the expression of about 25% of all yeast genes are correlated with the rate at which the cell is doubling regardless of what nutrient it is limited for[12]. Recent studies have characterized modules of physiological responses that are associated with 'proper' nutrient regulation, known as the growth-rate response (GRR)[13]. Positively correlated (higher expression with faster growth) genes of the GRR are associated with protein synthesis and mitochondrial function while negatively correlated genes of the GRR are associated with stress response, and autophagy[13]. The phenotypic consequences of this second situation, one where growth is difficult, are also distinct: yeast cells lower the consumption of glucose and (in the case of total nutrient starvation) arrest in G0/G1[14,15]. These modules tell an intuitive story: when cells can grow abundantly, resources are used to make replicate, synthesize, and divide and when it cannot resources are conserved and defense genes are turned on to promote survival.

Given the strict response that yeast has to nutrient availability, the expectation would be that regulation at the genetic level would be optimized through evolution to allow for the most control possible without sacrificing robustness. One such pathway is the Target Of Rapamycin (TOR) pathway, which is conserved between yeast and humans[16].

1.3 TORC1 IS ESSENTIAL FOR NUTRIENT SENSING IN YEAST

The TOR pathway is seen as a 'master regulator' of nutrient sensing and can incorporate many unique metabolic inputs, including glucose[17], nitrogen[18], and carbon-fixed sulfate compounds (known as organosulfur compounds)[19]. The yeast genome encodes for two different proteins, Tor1 and Tor2, which form distinct multi-protein complexes. Tor1, in conjunction with Kog1, Lst8, and Tco89 make up the TOR1 complex (TORC1). TORC1 is thought to be the key sensor in nutrient availability: when nutrients are available, TORC1 is on and signals for processes like ribosome biogenesis and nutrient uptake to be on while signaling for stress response processes, such as autophagy, to be turned off. TORC1 is also shown to be sensitive to rapamycin, which leads to TORC1 inhibited processes, like autophagy, to be turned on. A simplified diagram of this is shown in Fig. 1. Tor2 complexes with Avol-3, Bit61, and Lst8 to form the TOR2 complex (TORC2), which is rapamycin insensitive and thought to modulate other growth processes like cytoskeleton development[16]. Much of the physiological responses associated with 'proper' nutrient regulation are thought to be associated with proper TORC1 signaling[14].

1.4 SOME YEAST AUXOTROPHS BYPASS TORC1

The tight regulation of TORC1 on nutrient availability is suggestive of evolutionary pressure to properly monitor and respond to various levels of nutrients like glucose, nitrogen, and organosulfur. However, it has recently been demonstrated that yeast are not optimized to properly regulate limitation for all nutrients. Studies with yeast *auxotrophs* (a strain that cannot make an essential compound such as an amino acid or nucleotide) demonstrated markedly different physiological responses to limitation for the cognate metabolite. For example, in the

presence of abundant glucose, uracil and leucine auxotrophs proceed to waste glucose due to failure to regulate fermentation[12]. This wasteful phenotype leads to a rapid loss of viability under starvation for the required metabolite[4].

This pattern has also been observed for lysine auxotrophs of yeast limited for lysine but in the abundantly supplied with other natural metabolites like glucose[14]. In addition to the wasteful glucose use and poor viability, gene expression analysis demonstrated that many modules that should be turned on under conditions of nutrient limitation, such as autophagy and stress response genes, were off. Conversely, gene modules that should ideally be turned off, such as ribosome and protein synthesis genes, were on. Interestingly, these auxotrophs with poor viability could be rescued by inhibiting TORC1 signaling, either with rapamycin or through gene knockouts[4].

These data taken together suggest that certain amino acids, such as a lysine, have not been constrained by evolution to be sensed by TORC1, but rather bypass TORC1 entirely. In the presence of abundant natural metabolites like glucose, TORC1 is singled to be on and proceeds to dictate cellular growth. However, if this same strain with TORC1 on, is limited for lysine, the cell proceeds as if sufficient nutrients are available. This results in glucose wasting, aberrant synthesis of ribosomes and related genes, and eventual cell death.

1.5 How would a Misregulated Nutrient Sensing System Adapt?

The lack of evolutionary pressure to constrain lysine auxotrophs to a 'proper' physiological response under lysine limitation creates a system to study how a misregulated nutrient sensing system, where the master regulator TORC1, is bypassed can adapt to this aberrant phenotype. Understanding how such a system could adapt over time to compensate for

this misregulation is relevant to understanding fundamental questions not only of biological regulation of metabolism, but also human diseases. Many clinically characterized cancers are believed to have severe misregulation of TOR signaling[20]. Therefore, a yeast lysine auxotroph growing under lysine limitation could be used as a model to understand how tumors could adapt over time. Additionally, the selective pressure to mitigate such an aberrant phenotype could yield insight into possibly non-intuitive evolutionary solutions.

Therefore, for my thesis, I used experimental evolution to study how a lysine auxotroph of yeast (herein called **[L-]**) could adapt to limitation for lysine. The ancestral strain shows the expected phenotypes consistent with the model presented in Fig. 1. The strain dies when given glucose but not lysine (TORC1 is on when it shouldn't be), lives when given lysine but not glucose (TORC1 is off when it should), and lives when given glucose, no lysine, and treated with rapamycin (TORC1 is off when it should). These data are summarized in Fig. 2.

In Chapter 2, I will outline initial findings of this experiment. As expected, the first wave of adaptive mutations correspond to enhanced scavenging for lysine, consistent with many studies of yeast grown under nutrient limitation[21]. Surprisingly however, I find that roughly 10% of all the clones of had become auxotrophic (also known as *metabolically dependent*) for additional compounds that were not exogenously supplied in the media. These new auxotrophs in the **[L-]** lineage corresponded to either glutamine or organosulfur. I then will detail how this evolution was possible by 1) determining the source of nascent metabolites needed to support auxotroph evolution and the potential causes of this metabolite source emergence and 2) elucidating the evolutionary mechanism by which these auxotrophs rose to high frequencies in my lines.

In Chapter 3, I will identify glutathione as an important molecule in the nascent organosulfur niche. I will also show, using quantitative metabolomics, that glutathione is likely a predominant compound and possibly a foundation for the entire organosulfur niche. Additionally, I will show a striking relationship between the degree of lysine limitation and the release rate of glutathione from live cells.

In Chapter 4, I will demonstrate that organosulfur auxotrophy is adaptive and can be supported in a negative frequency-dependent manner by organosulfur prototrophs (strains that can make organosulfur). This adaptive benefit is not due to more efficient growth or energy savings, but rather by enhanced viability. I will show that this is likely through restoration of TOR signaling.

In Chapters 5 and 6 I will discuss these findings in the context of their contribution both to understanding regulation of metabolism, but also how this study can provide insight into evolutionary and ecological processes to better understand microbial ecology.

1.6 Materials and Methods

Microscopy Experiments

The methodology for these experiments will be outlined in detail in Chapter 3. For the experiment showing differential viability of the **[L-],** WY2490 was grown overnight to exponential phase in YNB+2% glucose+164 μM lysine, washed 3 times in YNB+2% glucose, and starved for 3 hours at 30 ^{o}C in a never-used 13 mm test tube in YNB+2% glucose. Then, the cells were harvested, washed 3 times with YNB (no glucose), and starved for 3 days at 30 ^{o}C. This was done to deplete the vacuole of stored glucose. In a 96 well plate with either YNB, YNB+2% glucose, or YNB+2% glucose+1 μM rapamycin, ~10,000 cells/well were inoculated and mCherry intensity over time was collected. Data were normalized to starting intensity and

plotted in Fig. 2. In cases where death rate was assessed, normalized fluorescence intensity was plotted against time for the entire experiment. A decrease in fluorescence intensity has been shown by our group to correlate well with cell death (data not shown).

1.7 FIGURES

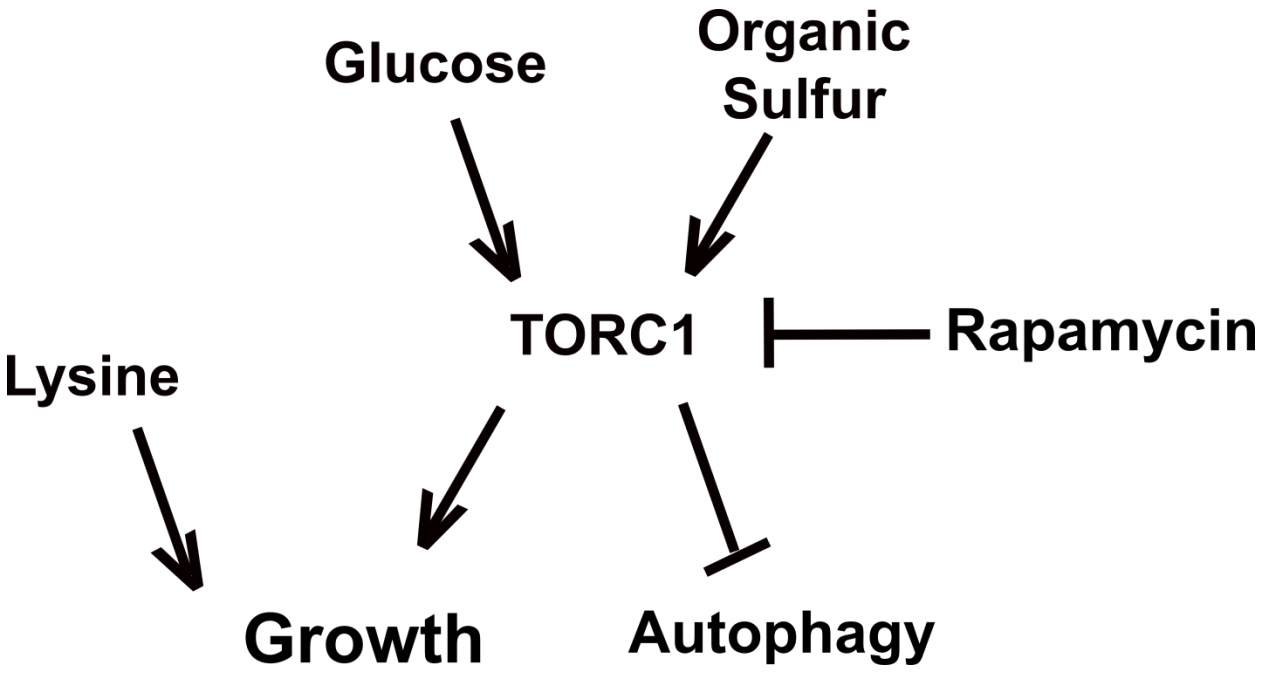

Figure 1- A Simplified Diagram of TORC1 Signaling.

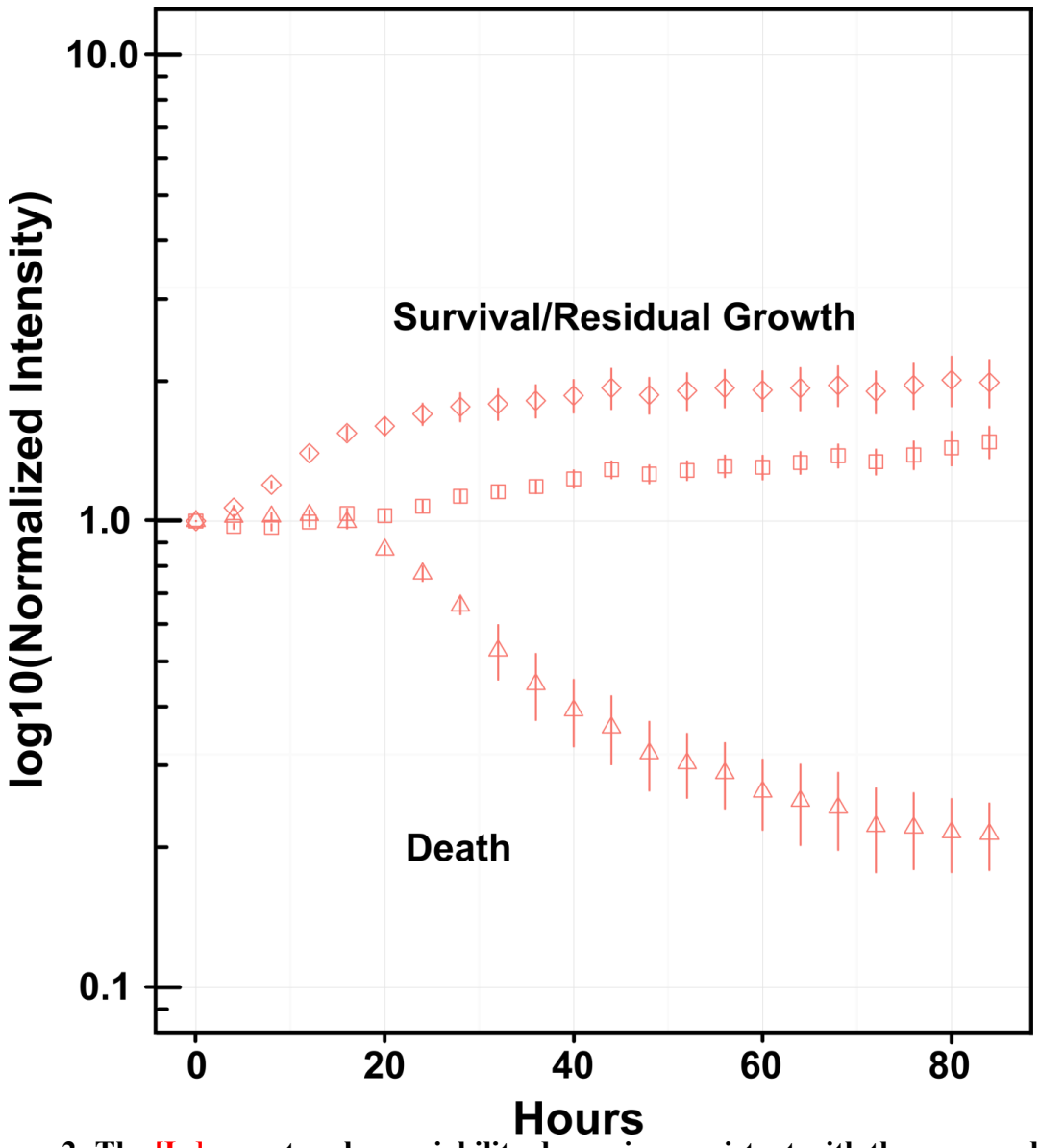

Figure 2- The [L-] ancestor shows viability dynamics consistent with the proposed model of misregulated TORC1. Triangles correspond to **[L-]** in YNB+2% glucose, lacking lysine. Squares correspond to **[L-]** in YNB (no glucose) with 164 µM lysine. Diamonds correspond to **[L-]** in YNB+2% glucose, lacking lysine, but in the presence of 1µM rapamycin. The **[L-]** was grown to exponential phase, starved for lysine and glucose to deplete vacuolar stores, and imaged over ~90 hours. Decrease in fluorescence intensity corresponds to death while maintenance of fluorescence corresponds to staying viable. The **[L-]** dies when TORC1 is on in the absence of lysine but lives when TORC1 is off, as expected. Error bars correspond to 2 standard deviations of average normalized mCherry intensity for 5 replicates/wells.

Chapter 2. EVOLUTION OF AUXOTROPHY FOR METABOLITES NOT SUPPLIED EXOGENOUSLY

2.1 OVERVIEW OF EVOLUTION EXPERIMENT

I wanted to study how yeast could adapt to an 'unnatural' state of nutrient limitation. Given that metabolism is so tightly regulated (see above chapter) and auxotrophy for an 'unnatural' metabolite such as lysine was not the same as limitation for a 'natural' nutrient like glucose, I hypothesized that suppressor mutations would likely emerge to mitigate the wasteful metabolism of these auxotrophs. To test this, I evolved a lysine auxotroph of the budding yeast *S. cerevisiae* (herein referred to as the ancestral **[L-]** strain) under two different conditions of lysine limitation. Briefly, **[L-]** was grown in a relatively constant environment of limited lysine for 50-150 generations (see Materials and Methods for further details) (Fig. 1). In this case, 'limited lysine' refers to an environment where growth rate of **[L-]** follows Monod kinetics below maximal growth rate [22]. Lysine limitation was imposed in one of two ways: either in a controlled environment of a chemostat [11] or by coculturing the **[L-]** strains with a lysine over-releasing strain [23] in a cross feeding system known as CoSMO (Cooperation that is Synthetic and Mutually Obligatory). In this case, the co-culture relies on each partner for the required metabolites, so the population can be thought of as in a state of 'partner-imposed' nutrient limitation. Throughout the experiment, populations diverged from the ancestral **[L-]** genotype through the acquisition of novel mutations, resulting in heterogeneous populations. Periodically, these heterogeneous populations were harvested and frozen down in trehalose for further analysis.

The logic for this evolution experiment is that the low lysine levels the **[L-]** strain would evolve under would create two selective pressures: one to scavenge as much lysine as possible and one to mitigate the misregulated TORC1. The low viability that the **[L-]** strain has under limitation for lysine (Fig. 2) presents an evolutionary opportunity to suppress this phenotype and gain a possibly significant fitness advantage.

2.2 Clones with Enhanced Affinity For Lysine Rapidly Emerge

For all sequenced strains, clones carried mutations in either *ECM21*, *RSP5*, or *DOA4* which have previously been identified by our group as highly adaptive for the **[L-]** lineage in a lysine limited environment[24]. Rsp5 is an E3 ubiquitin ligase that has been shown to target the high-affinity lysine permease Lyp1 for degradation[25]. This process is facilitated by the arrestin-like chaperon protein Ecm21[26]. Doa4 also mediates the above process. Loss of function to either of these proteins results in stabilization of Lyp1 on the plasma membrane and dramatically improves the Km (Monod affinity constant) for lysine, allowing cells to grow at lower concentrations. Additionally, many strains sequenced showed a duplication of chromosome 14 (annotated as supercontig 1.7 for the RM11 assembly), where *LYP1* is encoded and has also been shown to be adaptive under lysine limitation for the **[L-]** lineage[26]. I have previously demonstrated that strains carrying these mutations can rapidly dominate a population of the ancestral **[L-]** strain: evolved strains with higher lysine affinity can make up to ~10% of the population within the first 24 hours of evolution (data not shown).

2.3 Auxotrophs for Metabolites Not Exogenously Supplied Emerge

When clones from these populations were isolated for further analysis, I observed that some (~10% of all tested) failed to grow in minimal media (Yeast Nitrogen Base+ 2% glucose,

see Materials and Methods, sometimes referred to as SD media) supplemented to 164 μM lysine final concentration, whereas the ancestral [**L-**] grew to saturation in this medium. Whole-genome sequencing of these clones revealed putative loss of function mutations in genes previously demonstrated to be required for amino acid synthesis (see Table S2 and Figure 1 for spotting).

Interestingly, auxotrophies seemed to be restricted to two specific types: organosulfur compounds (cysteine, methionine, AdoMet, homocysteine, glutathione, etc.), and glutamine. Organosulfur auxotrophs showed the highest prevalence in all clones tested. The evolution of auxotrophy was not strain dependent, as I isolated auxotrophs of **[L-]** lineages evolving under lysine limitation for both the S288C and RM11 backgrounds. These data, taken together, that indicate the auxotrophs for compounds not exogenously supplied by an experimentalist can readily emerge to high frequencies in lysine-limited populations, at least for the case of *S. cerevisiae.*

Of the 5 organosulfur auxotrophs sequenced, 4 contained loss of function mutations (nonsense or frameshift mutations) in the sulfur assimilation pathway[27], which is responsible for importing sulfate from the environment and reducing it to hydrogen sulfide, which is then incorporated into homocysteine through a branch point reaction with O-Acetyl-L-homoserine. Homocysteine is then used a precursor for the amino acids methionine and cysteine as well as similar compounds AdoMet and glutathione[27]. Hence, even these auxotrophs carry mutations in gene names annotated at *"MET"*, they are not simply methionine auxotrophs: any organosulfur compound downstream of the branch point reaction should theoretically support these strains provided they can be taken in by the cell (although there is only experimental evidence for methionine, cysteine, AdoMet, and glutathione).

I also wanted to see if I could infer anything about the evolutionary trajectories that gave rise to these auxotrophs. As part of our initial sequencing, I also sequenced strains from the same population that were not organosulfur auxotrophs. Satisfyingly, all of these clones carried identical point mutations in *ECM21* and *RSP5* as their corresponding organosulfur auxotroph line mates. These data, combined with the previous observation of the rapid dominance of strains carrying these mutations, suggests a simple scenario for auxotroph establishment: first the **[L-]** lineage is dominated by an *ecm21, rsp5, doa4*, and/or aneuploidy for chromosome 14 background. Next, an auxotrophy mutation is acquired in this background and rises in frequency in the population.

2.4 Determining How Auxotroph Evolution Was Possible

While the above scenario is intuitive, it is not complete. There are two additional questions that must be addressed: 1) where is the nascent supply of metabolites supporting auxotroph evolution and 2) how did a newly evolved auxotrophic background rise to such easily detectable (~10%) frequencies in the evolving **[L-]** lineage? Each of these questions is fundamental to understanding these phenomena, so I decided to address each separately. The next chapter will address the source of metabolites supporting auxotrophs evolution and address how the lysine-limited environment drove a proportion of the **[L-]** lineage to release metabolites. The following chapter will address how these auxotrophs could increase in frequency through a selective advantage mediated through reacquisition of TOR signaling. For the purposes of this study, I have restricted my analysis to organosulfur auxotrophs.

2.5 MATERIALS AND METHODS

Evolution Experiment

To evolve **[L-]** in a coculture, partners were grown to exponential phase in minimal SD medium (6.7 g/L Difco Yeast Nitrogen Base w/o amino acids w/ ammonium sulfate, 20 g/L D-glucose) supplemented with lysine (164.3 μM) and adenine sulfate (108.6 μM), respectively [28]. The cells were washed 3 times with SD, counted using a Coulter counter, and inoculated at various ratios to a total density of $5x10^5$/ mL. Three 3 mL coculture replicates per initial ratio were initiated. The cocultures were grown at 30°C in glass tubes on a rotator to ensure well-mixing. Optical density (OD) was measured in a spectrophotometer once to twice every day to keep a record of total generations. In this study, 1 OD600 was found to be $2\sim4x10^7$cells/ mL. Cocultures were diluted periodically to maintain OD600 at below 0.5 to avoid additional selections due to limitations of nutrients other than adenine or lysine. The populations were never diluted more than 20-fold to ensure no large population bottlenecks were experienced.

For every coculture at every 10~20 generations, a sample was subjected to flow cytometry to measure population composition and the total cell density; the cell pellet of ~1 mL coculture was resuspended in 1 mL YPD [28] + 10% trehalose, precooled at 4 ^oC for several hours, and frozen at -80 ^oC. To revive a coculture, ~20 μL was scooped from the frozen sample using a sterile metal spatula, diluted ~10-fold into SD, and allowed to grow to moderate turbidity for 1-2 days. The coculture was further expanded by adding 3 mL of SD. **[L-]** evolved clones were isolated by plating the coculture on rich media (YPD) agar with Hygromycin B, which the **[L-]** clones are resistant to.

Chemostat vessels were placed into a core manifold with six receptacles (Figure 2), each with a magnetic stirrer. Reactor vessels were immobilized in receptacles by adjustable

compression rings. A rubber stopper equipped with an inflow tube and a sampling needle covered the top of the vessel. Waste flowed by gravity to a waste receptacle below the device through C-Flex 0.375" ID tubing (Cole Parmer) attached to the outflow arm. Nutrient media was fed to the vessels from media reservoirs by tubing passed through a Cole Parmer MasterFlex C/L peristaltic pump controlled by a custom LabView console through a custom relay box. Media reservoirs were 1 L glass bottles capped with one-hole rubber stoppers. Tubing was fed through the stopper and allowed to hang to the bottom of the reservoir. The reservoirs were placed on Denver Instrument XP-1500 digital balances, and the actual flow rate for each vessel was determined from the rate of mass loss of the corresponding reservoir.

To create a sterile environment, initial assembly was done in autoclave trays, with vessels held in tube racks. Six reservoirs were prepared by adding 810 mL water to each bottle. Six vessels were prepared by adding a 10 mm stir bar and 20 mL growth media (SD+21 μM lysine) to each vessel. Media delivery tubing was attached between reservoirs and vessels through rubber stoppers, and waste tubing was attached to each outflow arm, with the unattached end covered by foil held in place by autoclave tape. A 1.5 mL micro-centrifuge tube was placed over the sampling needle and held in place by autoclave tape. Tubing ports were wrapped with foil as well. Each reservoir with its attached tubing was weighed, the entire assembly autoclaved, then each reservoir weighed again. Lost water was calculated and added back. Under sterile conditions, 90 mL of 10X SD and a lysine stock were added to each reservoir to reach a final lysine concentration of 21 μM. The vessels were then secured into the chemostat manifold receptacles, reservoirs placed on the scales, and tubing threaded into the pumps.

To inoculate the chemostat vessel, the **[L-]** ancestor was grown to exponential phase in SD supplemented with 164 μM lysine, washed with SD and diluted to OD_{600} of 0.1 (~$7x10^6$/

mL) in SD. 20 mL of diluted culture was added to each vessel through the sampling needle, followed by 5 mL SD to rinse the needle of excess cells. Of six total chemostat vessels, each containing ~43 mL running volume, three were set to operate at a target doubling time of 7 hours (flow rate ~4.25 mL/hr), and three were set to an 11 hour target doubling time (flow rate ~2.72 mL/hr). With 21 μM lysine in the reservoir, the target steady-state cell density was $7x10^6$/ mL. There was a variance of roughly 2-fold around the target cell density.

The nutrient reservoir was refilled when necessary by injecting media through a sterile 0.2 micron filter through a 60 mL syringe. To take samples sterilely, the covering tube on the sampling needle was carefully lifted, and a sterile 5 mL -syringe was attached to the needle. The needle was then wiped with 95% ethanol, and slowly pushed down so that the tip was at least ~10 mm below the liquid level. A 5 mL sample was drawn into the syringe, the needle pulled up above the liquid surface, and an additional 1 mL of air drawn through to clear the needle of liquid residue. The syringe was then detached and the cap placed back on the needle. The samples were ejected into sterile 13 mm culture tubes for freezing and flow cytometry determination of live cell densities.

Auxotroph plating

Isolated clones from evolution experiments were diluted into 1:6000 from a YPD stock culture into 3 mL of YNB (0.67% (wt/vol) Difco Yeast Nitrogen Base without amino acids, 2% (wt/vol) dextrose) supplemented with amino acids. Cultures were grown at 30 ℃ for at least 16 hours to bring cells to exponential phase growth where maximal growth rate (~ 0.5 doublings/hour, ~2 hour doubling time) was achieved. OD600 of cultures were tracked for at least one generation to ensure exponential phase growth was reached. When populations reached OD600 ~0.1-0.3 (7-21 x 10^6 cells/ mL), cultures were washed 3 times with YNB+2%

glucose without amino acid supplementation and recultured in 3 mL of YNB+2% glucose without amino acid supplementation and starved for 3 hours at 30 ℃. This was done to deplete vacuolar storage of amino acids. Additionally, all starvation was done in never-used glass test tubes to prevent possible nutrient contamination. During this time, ~0.5 - 1 doubling of residual growth was typically observed. After this time, OD600 was measured again, populations were diluted to 100 cells/μL, and 10 μL (1000 cells) were spotted onto YPD Agar, YNB Agar supplemented with lysine (164 μM), and YNB with lysine and additional amino acids. Population spots were grown for 2 days at 30 ℃before being analyzed.

Genomic Analysis

High-quality gDNA was extracted using the QIAGEN Genomic-tip 20G kid (CAT No. 10223) or the Zymo Research YeaStar Genomic DNA Kit (CAT No. D2002). Illumina libraries were prepared using a Nextera DNA Sample Preparation Kit with 96-custom barcode indices [29] and TruSeq Dual Index Sequencing Primers. Libraries were pooled and multiplexed on a HiSeq2000 (Illumina) for 50 or 150 cycle paired-end reading. A custom genomic pipeline written in Perl that incorporated the bwa aligner [30] and samtools[31] for alignment file generation, GATK for SNP/indel calling [32], and cn.MOPs for local copy-number variant calling [33]. Ploidy calculated using custom python and R scripts that analyzed mean-normalized coverage across each chromosome or supercontig relative to the coverage of the entire genome. Finally, a custom Perl script using vcftools [34] was used to automate the comparison of an evolved clone with its evolutionary ancestor. All called mutations were validated by visual inspection in the IGV environment[35].

2.6 FIGURES

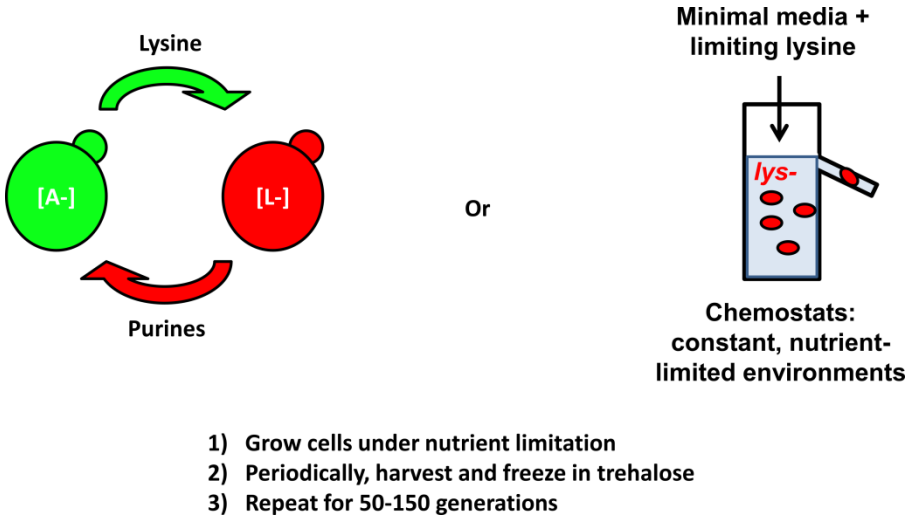

Figure 3- Summary of Evolution Conditions. Populations of the **[L-]** lineage were evolved under lysine limitation to determine how adaptation to an unnatural metabolic state could occur. This was done in a cross-feeding coculture of serial batch transfer (left) or in controlled environment of a chemostat (right). Populations were periodically frozen in trehalose to maintain a fossil record of the experiment for further analysis.

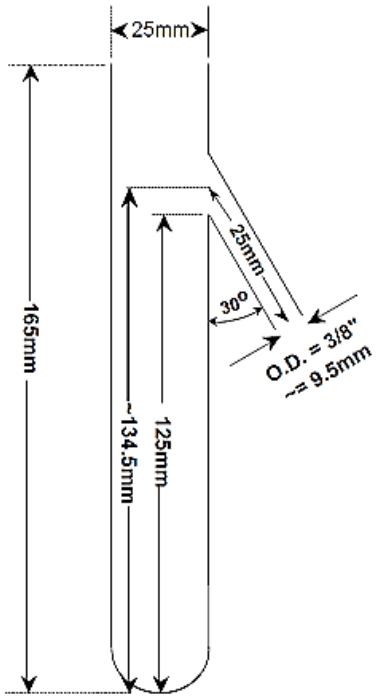

Figure 4- Chemostat Vessel Description

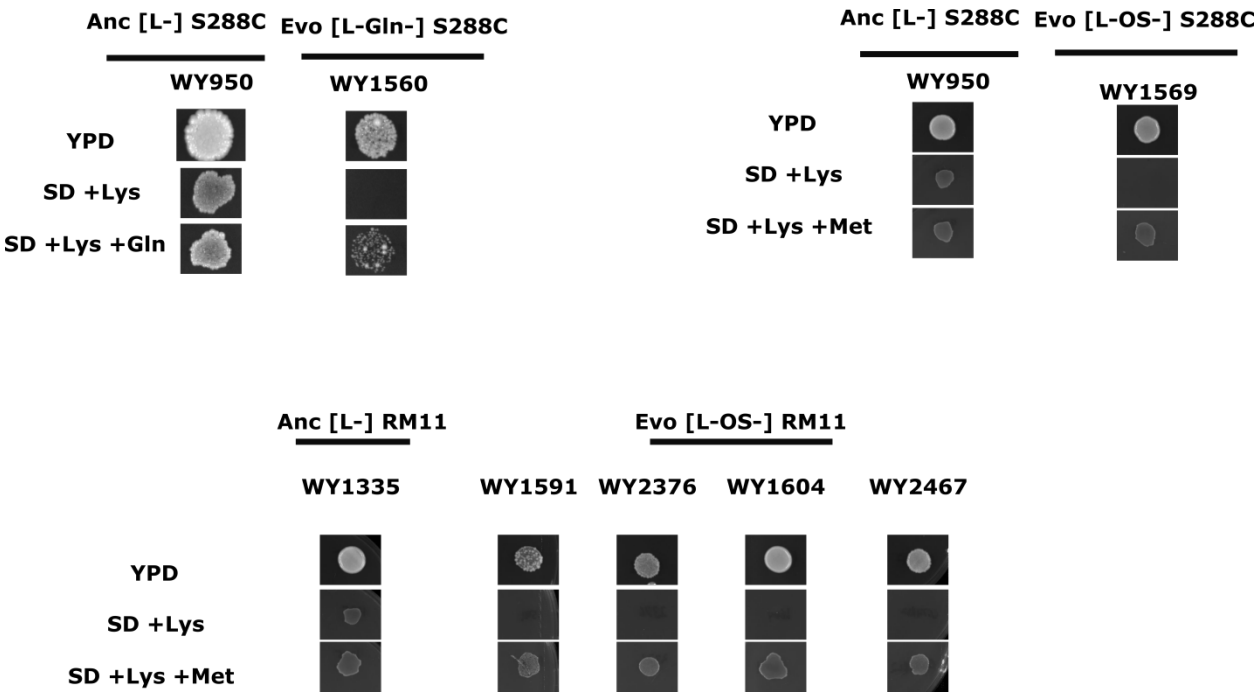

Figure 5- Evolution of Auxotrophies for Metabolites Not Supplied Exogenously. The clones tested were grown exponentially, washed free of supplements, and starved for at least 3 hours to deplete vacuolar stores of amino acids and peptides. Cultures were then spotted onto the media agar outlined above. All evolved clones shown here fail to grow without supplementation for glutamine or organosulfur. These growth defects are restored upon re-supplementation with glutamine or organosulfur.

Chapter 3. LYSINE LIMITATION DRIVES THE RELEASE OF GLUTATHIONE FROM LIVE CELLS

3.1 A NICHE THAT CAN SUPPORT ORGANOSULFUR AUXOTROPH GROWTH IS RAPIDLY CONSTRUCTED BY THE [L-] LINEAGE IN RESPONSE TO LYSINE LIMITATION

I first sought to address the nascent supply of metabolites that could support auxotroph evolution (hereafter referred to as a 'niche'). Since the required niche was not provided exogenously during the evolution experiment and organosulfur auxotrophs emerged in chemostats, I reasoned that the **[L-]** lineage (or a sub-population of it) must be releasing the metabolites into the media, thus providing the evolutionary opportunity for auxotroph emergence. This process, known as *niche construction*, is widely observed both in nature[36] and experimental evolution studies[37] . Notably, when *E. coli* is evolved under glucose limitation in a chemostat, a glucose specialist emerges that has a greater affinity, but as a side-effect also releases acetate which provides a niche to support a newly evolved acetate specialist[37]. Additionally, during the famous Lenski LTEE, there is stable co-existence of two ecotypes: an L ecotype which feeds off of some compound released by a S ecotype[38]. Recent deep sequencing analysis of the Lenski lines also showed pervasive coexistence of genotypes, seemingly through negative frequency-dependent fitness tradeoffs mediated by cross-feeding [39]. In all of these cases, the emergence of the important niches occurred after a significant amount of evolutionary time (many generations), indicating that selection and adaptation were required to produce an evolved genotype with the necessary niche-constructing capabilities. Another possibility is that the ability to construct niches is also present in the ancestor of an evolution experiment and, over the course of adaptation; this ability is retained or modified. To test this, I grew the **[L-]** strain for

24 hours in the environment it evolved in during the original experiment (lysine limitation) in a chemostat (see Materials and Methods). Periodically I sampled from the chemostat vessel and filtered the supernatant. I then asked if an organosulfur auxotroph could grow in the spent media (See Materials and Methods). Satisfyingly, supernatant harvested from 12-24 (<4 generations) hours can support auxotroph growth (Fig. 6). Because of the short time scale of the experiment, I concluded that the ancestral genotype of the **[L-]** lineage carried the ability to construct the organosulfur niche.

3.2 GLUTATHIONE IS THE PREDOMINATE COMPOUND OF THE ORGANOSULFUR NICHE

I considered three main questions for further analysis:

1. What compound(s) makes up the niche supporting the organosulfur auxotrophs?
2. How is the compound(s) being exported into the environment? Cell lysis or a physiological process?
3. What is the driving force behind the release of this compound(s)?

In order to answer these questions, I first sought to identify the compounds of interest that made up the organosulfur niche. To test this, I grew the ancestral **[L-]** strain in varying degrees of lysine limitation (by permuting the doubling time of the chemostat, see Materials and Methods) and assessed the relative pool sizes of organosulfur compounds using a growth-rate based bioassay. The logic was that if there was a difference in pool size across different lysine conditions then I could use that to identify compounds that fit the same pattern by metabolomics methods (such as mass spectrometry) to identify candidates that make up the niche. I initially observed that, using a growth rate quantification assay (See Materials and Methods), there seems

to be a ~2-fold difference in niche size, with a greater amount of organosulfur compounds present in the 8 Hr doubling chemostat supernatants, when comparing the supernatants of the ancestral **[L-]** lineage grown under an 8 hour doubling and a 4 hour doubling (Fig. 7).

Next, I performed liquid-chromatography mass spectrometry (See Materials and Methods) on these supernatants and specifically looked for target ions that were indicative of the previously published compound shown to support organosulfur auxotrophy (methionine, cysteine, glutathione, AdoMet, and homocysteine) [27]. Only glutathione in the reduced/monomeric form (GSH) was detected in our supernatants (Fig. 7) and resembled the expected pattern of differing concentration across different sets of supernatants.

To validate this finding, I constructed a glutathione auxotroph (*gsh1::KanMX*) and asked if it could grow unsupplemented in our supernatants. This strain did indeed grow (Fig. 9) indicating that glutathione was present. I next sought to determine if glutathione was the predominant compound that made up the organosulfur niche. A brief analysis of the KEGG database of yeast metabolism indicates that there are >100 organosulfur compounds that, if they could be imported, should theoretically be able to support an organosulfur auxotroph. Rather than screening exhaustively for all these metabolites, I took a gene-centric approach. In yeast, in order for an organosulfur auxotroph to utilize glutathione as an organosulfur source, it must cleave away the internal cysteine amino acid using the peptidase Dug1[40]. Therefore, a *dug1* organosulfur auxotroph is unable to use glutathione to fulfill its auxotrophic requirement. This strain fails to grow to any detectable optical density in our supernatants without supplementation of methionine (Fig. 9 and Fig. 17). I tested the growth of this strain under a range of methionine (an organosulfur source that can be used) and found that this strains grows to easily detectable densities at ~1 μM (Fig. 17). This leads me to hypothesize that any additional compound beyond

glutathione must be at a concentration of less than 1 μM. It is possible, however, that there are more abundant compounds present in the supernatant that my tester strain has poor affinity for. In section 3.6 I will address this point and possible experimental solutions.

Interestingly, when I grow the glutathione auxotroph in supernatant harvested from cells growing under lysine excess at the same cell densities (see Materials and Methods), the strain fails to grow at detectable densities. A characterization of the glutathione auxotroph yields under various concentrations of glutathione indicated that that this strain can grow to detectable densities at ~0.05 μM glutathione, indicating that the concentration of the glutathione in the supernatant harvested from the lysine excess conditions was lower than this.

3.3 There is a Quantitative Relationship between Lysine Limitation and Glutathione Release

I was intrigued by the possibility of differing glutathione pool sizes available to populations growing under differing degrees of lysine limitation. To test this, I developed a highly sensitive HPLC protocol based on a previously characterized fluorescent conjugate (See Materials and Methods). The assay sensitivity was 0.03 μM GSH in a 10 μL injection with a linear range up to 1 μM GSH (Fig 12). Next, I used this assay to quantify the GSH pool in supernatants harvested from populations taken from lysine excess (~1.6 hour doubling) to lysine-stressed (~8 hour doubling) populations harvested from the same founder population (See Materials in Methods). There appeared to be a ~10-fold difference in the GSH concentration (~0.03 vs ~0.5 μM) between the two populations. Normalizing by cell number and doubling time gives the net release rate of GSH per cell[41]. It appeared there was still a two-fold difference in release rate of GSH between the lysine-excess and lysine-stressed populations ($p < 1x10^{-5,}$ one-sided Welch's t-test). This indicates that the release rate of GSH may be a function of cell

physiology: more lysine stressed populations appear to release more GSH. Similar phenomena have previously been observed for both yeast[42] and other industrially relevant microbes[43].

3.4 Glutathione is Released By Live Cells

Why would greater lysine limitation cause greater release of GSH? In the trivial case, more lysine-limitation leads to more cell death and lysis - this causes greater pools of GSH to appear. Indeed, lysine auxotrophs of yeast in our hands show poor viability under lysine starvation (Fig. 2 and Fig. 21) and lysine-stressed populations have ~4-fold more dead cells compared to lysine-excess populations. Therefore, I needed to determine if the total amount of GSH that could theoretically be released by the total amount of dead cells in our population could theoretically explain all of the GSH I measured in our supernatants. To test this, I used an established metabolite extraction protocol[44] that collects a 'snap shot' of the metabolite pools inside a yeast cell. Briefly, populations are harvested and quickly filtered onto a nylon membrane, which is submerged into ice-cold organic extraction buffer. This makes the cell membrane permeable and quenches metabolism by lowering the internal temperature of the cell and denaturing enzymes in the cytosol. This extracted pool of metabolites can then be measured by HPLC and normalized to the total amount of harvested cells (See Materials and Methods for full description). This value gives the actual amount (fmole GSH per cell) of glutathione that can be contributed to a single cell assuming that each cell is represented by this population average. Next, I normalized the total pool of glutathione in the supernatant, determined by HPLC, against only the *dead cells* in my chemostats, which are determined by live-dead staining (see Materials and Methods). This number is the *theoretical* amount of glutathione each dead cell would need to release (fmole GSH per dead cell). Quantitative differences between this theoretical amount and the actual amount of glutathione each cell carries indicates that the construction of the

organosulfur niche cannot be explained by cell lysis alone e.g. live cells must be releasing glutathione as part of their physiology[42].

Figure 14 shows representative data from a typical metabolite extraction. There is roughly an 8-fold difference in the amount of glutathione that each dead cell would need to release and what is inside the average cell. Assuming each yeast cell is roughly 40 fL[45], I estimate the concentration of glutathione inside a yeast cell to be 2.5-4 mM, which is consistent with previously published values of 1-10 mM[46], indicating that my estimation is reasonable. Based on these data, I conclude that cell lysis alone cannot explain the glutathione pool in my chemostat experiments, indicating that there must at least a live-cell release component in response to physiological stress of lysine limitation. It should be noted that I am assuming that the average amount of glutathione is representative for each cell in the population (both live and dead). I am excluding the possibility that cells that are about to die rapidly accumulate glutathione in the cytoplasm, as there is no evidence in the literature to my knowledge that would suggest this.

3.5 Reduced Glutathione Quantified By HPLC Does Not Explain the Entire Organosulfur Niche. However, the Niche is Likely Glutathione-Based

While the identification of glutathione by mass spectrometry and subsequent quantification by HPLC does elucidate a mediator of this interaction, it may not necessarily be the whole story. It's possible that the **[L-]** releases several different compounds that make up the total organosulfur niche, but I haven't yet identified and quantified them. To test for this possibility, I developed a yield-based bioassay using an evolved organosulfur auxotroph (WY1604) to assess the total pool of organosulfur compounds in supernatants harvested from an

8Hr doubling time chemostat. This assay had a linear range of 0-5 μM glutathione and was sensitive down to 1 μM (Fig. 15. I used this assay to assess the total pool of organosulfur for the same supernatants that were quantified simultaneously for reduced glutathione by HPLC. Representative data are shown in Fig. 16. On average, my bioassay indicates that there is a total pool of organosulfur compounds is at a concentration of roughly 1.5 μM. This value is ~3-fold greater than my HPLC analysis that suggest that the concentration of reduced glutathione is ~0.5 μM. Taken at face-value, these data suggest that reduced glutathione composes roughly 1/3 of the organosulfur niche.

I wanted to see if there was an obvious explanation for this discrepancy between the bioassay and HPLC results. Previous data with a *dug1* strain, one that cannot use glutathione as an organosulfur source, suggested that there weren't any other compounds in high concentration (see Fig. 9). To follow-up on this result, I quantified the growth of this strain in various concentrations of a high affinity organosulfur source (methionine). I found that this strain can grow to easily detectable densities at concentrations as low as 1 μM organosulfur (Fig. 17). Since the difference between my HPLC measurement of reduced glutathione (0.5 μM) and my bioassay of total organosulfur (1.5 μM) is 1uM, the *dug1* strain is theoretically able to use the pool of organosulfur compounds for growth if the remaining 1 μM is not glutathione-based. The fact that this strain fails to grow at all in my supernatants would therefore suggest that the remaining 1μM of organosulfur must be glutathione-based. This would explain all my quantifications: the total 1.5 μM of organosulfur is all glutathione based, a third of which is reduced glutathione that can be detected by HPLC. However, given the previously discussed affinity issue discussed in Section 3.2, I would need more concrete experimental evidence to formally conclude that my entire niche is glutathione based.

Additionally, the possibility that the entire pool of organosulfur seems to be glutathione based reinforces the argument that cell lysis alone cannot explain the total pool of organosulfur: if a cell was lysing, why would only glutathione-based compounds be present in the supernatant? The fact that the entire pool of organosulfur could be glutathione based is striking: why would a particular family of compounds be so targeted for release by a cell? In section 3.6 I will speculate on the identity of these additional glutathione-based compounds.

3.6 Speculating on the Remaining Compounds in the Niche

The fact that the entire pool is possibly glutathione based is not necessarily surprising. Glutathione is biologically diverse molecule and highly abundant in the cell (1-10 mM in the reduced form)[46]. The free thiol group of glutathione is highly reactive[47] and as a result can for a diverse array of different molecules. Additionally, glutathione is a peptide and can form peptide bonds. Glutathione can exist in large polymers, called phytochelatins, and are shown to be abundant under conditions of heavy-metal stress[48]. Under conditions of heat stress, glutathione is known to react with proteins to form protein-SSG mixed disulfides, which protect against damage from the formation of disulfide bridges between proteins[48]. Finally, the ratio reduced and disulfide form of glutathione (GSSG), is known to be maintained as a redox buffer that protects against oxidative stress. The seeming ubiquity with which glutathione can react would suggest that systematic identification would be too onerous for one study, but also makes it reasonable to suggest the total pool of organosulfur compounds could be glutathione-based.

A simple experiment (currently on-going with collaborators) would be to treat supernatants or extracts with a reducing agent, such as dithiothreitol (DTT) or tris(2-carboxyethyl)phosphine (TCEP) , to reduce all the GS-X based compounds to GSH. Theoretically, any GS-X compound should be reduced to the GSH form, which could then be

quantified by HPLC or mass spectrometry. This is a simple experiment that may close the gap between my HPLC and bioassay results.

3.7 Nutrient Limitation Induced Niche Construction Explains the Metabolite Pool Required For Auxotroph Evolution

In order for organosulfur auxotrophy to become common in these evolving populations, there must be a source of metabolites that can support the nascent auxotroph with the compound they are unable to make. Organosulfur auxotrophy, conferred by acquisition of loss-of-function mutations in the sulfur reduction pathway are supported by a rapidly constructed pool of compounds that seems to be predominantly composed of glutathione in the reduced form. While yeast are known to excrete a variety of compounds[49] as normal physiological process, the high concentration of glutathione that allowed these genotypes to rise to high frequencies (~0.5 μM) is striking. I found that then environment of lysine limitation *itself* was driving the release of glutathione from live cells in a stress-dependent manner. The more limited for lysine cells became, the more glutathione they released. While the possible mechanisms have not been determined in this study (speculation below), the ecological implications are important to consider (fully discussed in Chapter 6). Ultimately, metabolic dependencies between microbes have been speculated to be common based on metagenomics data[50]. Additionally, several ubiquitous and well-studied classes of marine microbes, such as the SAR11 clade[51] and several species of green algae[52], are auxotrophic for amino acids and vitamins. A unifying factor of many environments, including these marine environments, is that they are likely nutrient-poor[53] , meaning that metabolic dependency in these ecosystems would not be the product of harvesting excess pools of nutrients, but rather from microbial trade[54]. The physiological reasons for *why* microbes would excrete various compounds are

therefore important. In Chapter 7, I will discuss the potential evolutionary consequences of stress-induced metabolite release.

3.8 Possible Explanations for the Effects of Lysine Limitation on Glutathione Release

Only recently has 'omics' technology been used to explore the relationship between genomics, physiology, and metabolite release, meaning that cellular mechanisms of lab-evolved interactions are rare to find. However, there have been successes in the literature. For example, a recent study of yeast and lactic acid bacteria found that yeast undergo nitrogen overflow in certain environments. To compensate for this, excess nitrogen is fixed in the form of amino acids and excreted by live cells into the medium, providing a niche to support metabolic dependency in lactic acid bacteria[55]. Genomics has also been used to identify causal genes responsible for ethanol release that supports cross-feeding between three ecotypes of *E. coli* in glucose limited chemostats[37]. Finally, genomics has also been used to pinpoint possible causes of succinate cross-feeding in the Lenski long term lines[56].

The cellular mechanism for the quantitative relationship between live cells releasing more glutathione under greater lysine limitation (see Fig. 13) has not been determined in this study. However, I will speculate as to possible mechanisms in this section, with the caveat that this is merely a set of different hypotheses.

Analysis of gene expression data from a previous study characterizing yeast lysine auxotrophs show that many genes are upregulated that can have ecological consequences [14]. For example, many relevant peptide transporters and amino acid synthesis genes are upregulated under severe lysine limitation relative to yeast starved for glucose. While the strain background between the previous work and my study is different, it is reasonable to conclude

that a similar phenomenon may be occurring. In this case lysine limitation could lead to an excess pool of amino acids. If these amino acids include cysteine, glutamate, and glycine, then these could be incorporated into glutathione. Additionally, lysine auxotrophs limited for lysine appear to favor fermentation over respiration. This could mean that the oxygen content of a cell is diminished and redox buffers such as glutathione are less important. This increased concentration, in addition to upregulation of oligopeptide transporters could provide enough of a thermodynamic 'push' that leads to glutathione excretion.

Another possibility is that cells experience redox stress under lysine limitation *because* of the excess concentration of glutathione. The free thiol group of glutathione may damage cellular machinery if present at too high a level. Indeed, it has previously been observed that yeast when treated with cadmium, which induces redox stress, rapidly excrete glutathione and upregulate glutathione transporters like Gex1[57]. Additionally, Opt1, an oligopeptide transporter shown to transport glutathione to the extracellular environment[58] appears to be upregulated in yeast in response to many different environmental stressors[59,60]. In both cases, the burden of lysine limitation could result in both an increase in the total pool of amino acids leading to glutathione production, which could favor glutathione release both by thermodynamics and by inducing glutathione transporters to export the high amount of glutathione.

Another possibility is that cellular fluxes of metabolites must be altered in order to create an optimal equilibrium under lysine limitation. Previous work for gene knockout libraries found that adaptive benefits of certain deletions can be attributed to redistribution of metabolite imbalance[61]. Flux balance analysis of metabolism predicted that various knockouts can be adaptive[61]. It's reasonable to imagine a scenario where adaptation to lysine limitation creates a metabolic imbalance, possibly due to upregulation of amino acid synthesis genes [14]. A way to

'solve' this imbalance could be the simple excretion of excess molecules, such as glutathione. This metabolic approach has been postulated to be pervasive in various *in silico* cross-feeding studies[62].

Finally, TORC1 has been implicated in yeast metabolite excretion[55]. Treatment of yeast with rapamycin induces a 5-fold increase in amino acid release[55]. While in this case TORC1 would be off, while in my studies TORC1 would be on, the link between TORC1 and metabolite excretion should be considered, given how pleiotropic TORC1 is. It's possible that misregulation of TORC1 can have effects on metabolite excretion.

Ultimately, the solution space for the quantitative relationship between lysine limitation and glutathione release is vast and out of the scope for this particular study. Future work could provide cellular and molecular insights, providing more evidence that diverse, cross-disciplinary approaches can be used to address new ecological questions in microbial communities.

3.9 Materials and Methods

Chemostat Experiments for Spent Media Analysis

Ancestral **[L-]** or derived clones (known as 'evolved') were grown in 50 mL YNB+2% glucose + 164 μM lysine for ~20 hours prior to inoculation. Before each experiment, growth was tracked to ensure cells were growing at optimally (~1.6 hour doubling time). When cells reached a density of ~0.2 OD600, cells were washed 3 times in SD and inoculated to a final cell density of $5x10^5$ cells/ mL in a chemostat vessel prefilled with YNB+2% glucose+21 μM lysine. This density corresponded to 5% of the carrying capacity of the chemostat of $1x10^7$ cells per mL. After this step, chemostat pumps were turned on at a set doubling time in a custom written LabView software package (see Chapter 2 Materials and Methods for more details). After this,

cells reached carrying capacity after ~ 8Hours and then entered a lysine-limited state. Cells entered a steady state of ~$1x10^7$ cells/ mL after 10 hours and maintained that density for the duration of the experiment. During this time, cells are likely in a constant physiology of lysine limitation[11]. Typically, cell densities differed by no more than 15% between time points. Periodically, 4 mL of supernatant was harvested and dispensed into a sterile 15 mL conical tube. Next, 300 µL of this cell sample was removed and kept on ice for flow cytometry analysis. The remaining 3.7 mL of supernatant was filtered through a 0.22 µM nylon filter into 500 µL aliquots and frozen at -80C. Each chemostat was sampled one at a time to prevent cells diverging from their physiology of the chemostat environment. For experiments with metabolite extraction, the chemostat vessel stopper was removed and 20 mL of sample was harvested. Due to the breaking of vessel sterility, this would mark the end of the chemostat experiment.

Flow Cytometry

Population compositions were measured by flow cytometry using a DxP10 (Cytek) with three lasers (405 nm, 488 nm, and 633 nm). Fluorescent beads of known concentration (as determined by hemocytometer) were added to determine cell densities. A final 1:20,000 dilution of TO-PRO was used for each sample to determine live and dead cell densities. Analysis using FlowJo software showed obvious clustering of live and dead cells in the RedFL1 channel, with dead cells having a RedFL1 signal of $>10^3$. Dead cell densities typically were never higher than 10% in all conditions tested. Previous work in our lab has shown that percentage of live cells matches viable cell counts by plating.

Yield-based bioassays

Various tester strains (see Table S1 for details) were used in this study, but preparation for all yield-based bioassays was the same. Strains were grown for ~16 hours in YNB+2% glucose and any required supplements. During this time, growth rate was tracked to ensure cells were doubling as expected (1.6 to 3 hour doubling depending on the strain/condition). After this time, cells were washed 3 times with 3 mL YNB+2% glucose and starved for at least 3 hours at 30C in 3 mL YNB+2% glucose in a never used 13 mm test tube (to prevent inadvertent nutrient contamination). This was done to deplete cells of vacuolar stores of amino acids. Finally, cells were inoculated into a final volume of 150 µL into a 96 well plate of either metabolite standards or supernatants, supplemented with to 1x lysine (164 µM) and 2% glucose. For each bioassay, roughly 1000 cells were used per well and plates were incubated at 30C for 2 days. After this, OD600 was measured using a Gen5 plate reader.

Rate-based bioassays

A mCherry tagged yeast strain auxotrophic for organosulfur (WY2035) was pre-grown in YNB+2% glucose + 164 µM Lys +134 µM Met and growth rate was tracked by optical density to ensure the cell was growing as expected. Next, cells were washed 3 times in YNB+2% glucose lacking supplements, and starved for at least 3 hours in never-used 13 mm test tubes (to prevent nutrient contamination). Cell density was measured again by OD, and cells were inoculated to roughly 1000 cells /well in a 96 well plate in a total volume of 300 µL. The well was filled with either known quantities of organosulfur (methionine or glutathione) in YNB+2% glucose or harvested supernatants supplemented to 2% glucose and 164 µM lysine so that growth rate should only be affected by organosulfur concentration. The microtiter plate was imaged periodically (1~2 hrs) under a 10x objective in a Nikon Eclipse TE-2000U inverted fluorescence

microscope using an ET DsRed filter cube (Exciter: ET545/30x, Emitter: ET620/60m, Dichroic: T570LP). For each well, 4 bins of 870 μM x 660 μM are collected in a grid shape. The microscope is connected to a cooled CCD camera for fluorescence and transmitted light imaging. The microscope harbors a temperature-controlled chamber set to 30^{o}C, and to maintain constant temperature, the air conditioning system of the room is turned off. The microscope is equipped with motorized switchable filter cubes capable of detecting a variety of fluorophores. It also has motorized stages to allow z-autofocusing and systematic xy-scanning of locations in microplate wells. Image acquisition is done with an in-house LabVIEW program, incorporating autofocusing in bright field and automatic exposure adjustment during fluorescence imaging to avoid saturation. To minimize the effect of evaporation, assay duration is generally within 24 hrs. In general, four locations per well are imaged, with ~20~80 initial cells per image. Time-lapse images were analyzed using an ImageJ plugin called Bioact3 written by Adam Waite. Foreground is selected by generating a binary mask. To create the mask, each bright field image is blurred with a Gaussian filter using a standard deviation (σ) of 1 pixel. Then, the rolling ball background subtraction algorithm is used to remove low frequency noise. The image is reduced to a bit-depth of 8, and each pixel is replaced by the maximum value in a 3 pixel radius. The resulting image is converted to a binary mask, holes are filled, and the mask is dilated by 2 pixels. From this mask, the percent pixels considered foreground is calculated. Each mask is then applied to the unmanipulated original image, and the foreground and background intensities are measured. The reported fluorescence is total fluorescence minus the background value calculated by the appropriate method. Previous analysis in the lab has demonstrated that fluorescence intensity correlates proportionally with cell density.

Reported fluorescence data is exported to a text via and analyzed via a custom R script.

For each well, fluorescent dynamics of each bin are plotted against time and inspected manually to identify possible faulty images (such as out-of-focus bins). If a faulty bin is identified, the time series of that bin is inspected manually for abnormalities and removed if any are identified. Typically, this is due to wells being out of focus due to autofocus on background particulate. Next, all bin intensities per time point are averaged. Next, fluorescent images are normalized to starting intensity (such that starting intensity is 1 and all subsequent intensity are the 1*the fold difference intensity). Max grow rate is calculated by measuring the slope of log10 (Normalized Intensity) against time. For each sliding window of 4 time points, slope is calculated and if it exceeds the current max slope for the well, it is chosen as the new maximum. To ensure that no estimation occurs when other metabolites, such as glucose, could be limiting, I restricted my analysis to data at 25% maximal intensity to ensure that cells had at least 2 doublings beyond when they are theoretically growing maximally. For this study, maximal growth rates were used to estimate approximate niche size, the logic being that cells should grow faster if there is a larger organosulfur niche.

Metabolite Extractions for Intracellular Glutathione Quantification

Metabolite extraction for chemostat populations was adapted from [44]. Briefly, 20 mL of chemostat populations are harvested in disposable 25 mL pipettes and rapidly vacuum filtered onto precut 0.45 μm Magna nylon filters (GVS Life Sciences, USA). Filters were the quickly submerged with ethanol cleaned forceps in into 3 mL ice-cold extraction buffer (held in a 5 mL sterile centrifuge tube) mixtures, which consisted of 40% (v/v) acetonitrile, 40% (v/v) methanol, and 20% (v/v) of distilled water. All reagents were HPLC grade and all extraction buffer mixtures were made fresh before each extraction. The centrifuge tube was capped and

quickly vortexed to remove all cells. The entire process took less than 25 seconds, with the time between populations being totally filtered and submerged in extraction buffer being less than 10 seconds. After all populations had been harvested, extracts were frozen at -80 C until solid, transferred to ice and allowed to thaw. After the samples had thawed, they were incubated on ice for 10 minutes and vortexed once every ~3 minutes and returned to -80C for refreezing (a single 'freeze-thaw' cycle). After 3 freeze-thaw cycles, 1.5 mL of sample was harvested and transferred to a new 1.5 mL micro-centrifuge tube, and centrifuged at 13,0000 rpm for 2 minutes at 4 C to pellet the cell debris. The extract was removed and the remaining cell extract/debris was added and spun down for collection. The final result was 3 mL of extracted metabolites that was stored at -80 C and analyzed by HPLC less than 48 hours after extraction. To check that a majority of metabolites were extracted, 100 µL of fresh extraction buffer was added to the collected cell debris, vortexed vigorously, and collected by centrifugation. This 100 µL 'second extract' was also analyzed for glutathione by HPLC. On average, the amount of glutathione in the second extract was < 2% of the amount extracted initially in the 3 mL extraction, indicating that the majority of glutathione was extracted.

Mass Spectrometry

Mass Spectrometry was performed by Wenyun Lu of the Rabinowitz lab at Princeton University. Supernatants and standards were shipped over-night on dry ice and analyzed immediately. Samples were analyzed using a Q Exactive Plus mass spectrometer coupled to Vanquish UHPLC system (Thermo Scientific). LC separation was achieved using a XBridge BEH Amide column (2.1 mm x 150 mm, 2.5 µm particle size, 130 Å pore size; Waters, Milford, MA) using a gradient of solvent A (20 mM ammonium acetate + 20mM ammonium hydroxide in 95:5 water: acetonitrile, pH 9.45) and solvent B (acetonitrile). Flow rate was 150 µl/min. The

gradient was: 0 min, 90% B; 2 min, 90% B; 5 min, 50% B; 10 min, 0% B; 13.5 min, 0% B; 15 min, 90% B; 20 min, 90% B. Column temperature is 25 ℃ and injection volume is 10 µL. Mass spectrometer parameters are: positive ion mode, resolution 140,000 at m/z 200, scan range m/z 290-650, AGC target 3E6, Maximum injection time 200 ms. Quantitation of Glutathione concentrations in samples were achieved by comparing the peak areas of glutathione in samples to those of standards in separate runs. Data were analyzed using the MAVEN software[63].

Glutathione Derivatization with Thiol Probe IV

Reduced glutathione was derivatized using a thiol specific probe first described by [47], called Thiol Probe IV (EMD Millipore) to make a fluorescent glutathione conjugate. The compound reacts readily with free-thiols, though at different rates (data not shown). For quantifying glutathione, 270 µL of sample or standard in YNB+2% glucose was added to 30 µL of 833 mM HEPES buffer, pH 7.8. This was done to raise the pH of the sample to a basic level, which facilitates the reaction. Next, the probe (dissolved in DMSO and stored in 50 µL aliquots at -20 C), was added to a final concentration of 100 µM, which is in excess of glutathione by at least 10-fold. The reaction was performed at room temperature in the dark (the probe is light-sensitive) in a 96-well plate for 20 minutes. After this, 8.4 µL of 2M HCl was added to rapidly quench the reaction by lowering the pH to ~2. This also stabilizes the fluorescent conjugate. Comparing HPLC traces of the same derivatized sample over 24 hours shows that glutathione peak area is within 10% of all replicates (data not shown). Following this procedure, the entire sample was added to a 250 µL small volume pulled point class insert (Agilent Part No: 5183-2085). The full sample volume of ~313 µL did to the top of the small volume insert for easy autosampler needle access. The small volume insert with sample was then placed inside a dark-

brown 1.5 mL autosampler vial (Shimadzu Part No: 228-45450-91) and capped with a fresh 9mm screw cap with PTFE septum (Shimadzu Part No: 228-45454-91).

High Performance Liquid Chromatography

Derivatized glutathione was separated and identified using reverse phase chromatography. 300 µL of sample was loaded into an Agilent 250 µL pulled point glass small volume insert (Part No: 5183-2085), which was then placed inside a Shimadzu 1.5 mL 12x 32 mm autosampler vial (Part No: 228-45450-91). 10 µL of the reaction mixture was injected onto Synergi 4 µM Hydro-RP 80 Å LC Column 150 x 4.6 mm (Phenomenex, Part No: 00F-4375-E0) fitted with a SecurityGuard Cartridges AQ C18 4 x 3.00 mm ID (Phenomenex, Part No: AJO-7511) in a SecuirtyGuard Cartridge Holder (Phenomenex, Part No: KJ0-4282). The SecurityGuard was replaced in the event of pressure reading exceeding the manufacturer's specifications periodically. Glutathione was eluted from the column with a mobile phase gradient of filtered Millipore water (Solution A) and acetonitrile (Solution B, HPLC grade). The Millipore water was filtered through a 0.22 µM filter prior to use. Additionally, before each run the column was equilibrated for 30 minutes with 1% Solution B. The % solution B followed the following program for each injection: 0 min 1%, 10 min 14%, 10.01 min 1%, and 15 min 1%. This corresponds to a gradual increase to 14% Solution B over 10 minutes, followed by a re-equilibration with 1% Solution B. The column was maintained at a running temperature of 25 ^{o}C in a Nexera X2 CTO-20A oven Flow rate was 1 mL /min. Under these conditions, glutathione eluted at ~ 7 minutes, with slight run-to-run variation. Fluorescent Glutathione was detected by excitation at 400 nm and emission at 465 nm. After each run, the column was washed and stored per the manufacturer's instructions.

Data Analysis

Analysis of HPLC data were done using the R Statistical Language with custom written software for peak-picking, baseline correction, plotting, and area estimation. Raw data for each sample run was exported to a text file and parsed in the RStudio environment. Emission data (at 465 nm) was culled to restrict analysis from 6.5 to 8 minutes. Next, my script identified a local maximal peak, which for concentrations about 0.01 μM glutathione always corresponding to glutathione. Anything lower and the signal was indistinguishable from background. Next, my script identified local minima on either side of the glutathione peak and drew a baseline that connected the two. The formula (y=mx+b) for this line was calculated and the baseline was 'corrected' by subtracting the emission spectrum value for each point against the y value of the calculated formula for the same point. An example of the steps of this pipeline is shown in Figure 11. To quantify the concentration of a glutathione, known concentrations of reduced glutathione in 1x SD media was subjected to the above procedure. A standard curve of 0.03 to 1 μM was typically used and showed little variability between experiments. An example of these data are shown in Figure 12. A linear regression model of peak area against concentration of reduced glutathione was built using the lm function of the stats package. Samples with areas within the dynamic range (0.03-1 μM GSH) were back-calculated using the linear regression model.

Calculation of Release Rates and Glutathione Content Needed for Dead Release

To calculate the release rate of glutathione per live cell, concentration of glutathione (in μM) was divided by live cell density (cell/ mL) as determined by TO-PRO straining (See Flow Cytometry section). This number was multiplied by $1x10^6$ to get fmole/cell. For each chemostat

population, the flow rate was calculated (/hr) and multiplied by the above number, resulting in fmole/cell/hr. This method assumes that the release rate of glutathione (or any arbitrary metabolite with a calculated release rate) is at steady-state and has previously been described by [41]. Comparing chemostat release rates quantified at different timepoints in the same experiment show good agreement (within 10%) after 22 hours of chemostat growth, which is where population dynamics also stabilize to chemostat dynamics (less than 10% variability in cell density between timepoints).

To calculate the amount of glutathione each dead cell would need to release for the total pool of glutathione to be explained by cell lysis alone (the 'dead release' model), concentration of glutathione (in μM) was divided by dead cell density (cell/ mL) as determined by TO-PRO straining (See **Flow Cytometry** section). This number was multiplied by $1x10^6$ to get fmole/dead cell. Assuming the TO-PRO staining is a fair proxy for total number of dead cells (independent experiments done in our lab suggest it is), this number is the amount each dead cell would need to have inside it to explain the total pool of glutathione by cell lysis alone. This method has previously been described by [42].

3.10 FIGURES

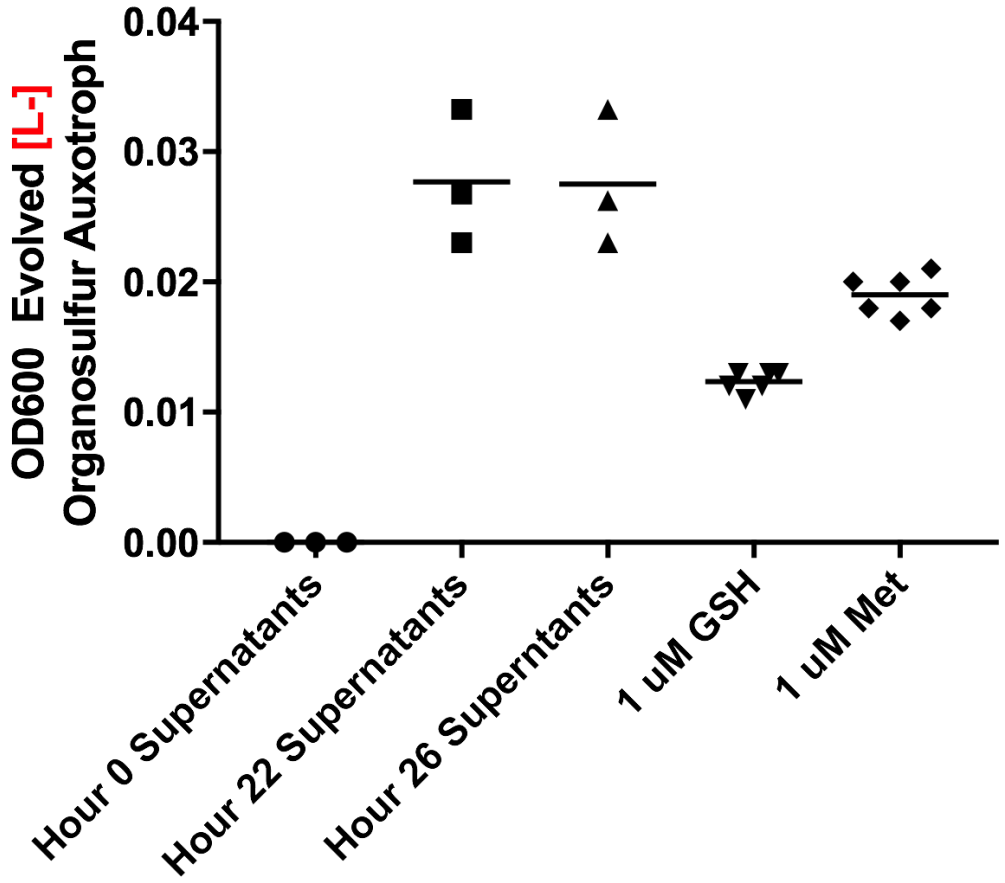

Figure 6 – A niche that supports organosulfur auxotrophs is rapidly constructed under lysine limitation. WY1604 (evolved organosulfur auxotroph in **[L-]** background) was grown to exponential phase, starved for lysine and used a qualitative screen for an organosulfur niche. Data are of final yield after 60 hours of growth. All supernatants were harvested from 8hr doubling chemostats.

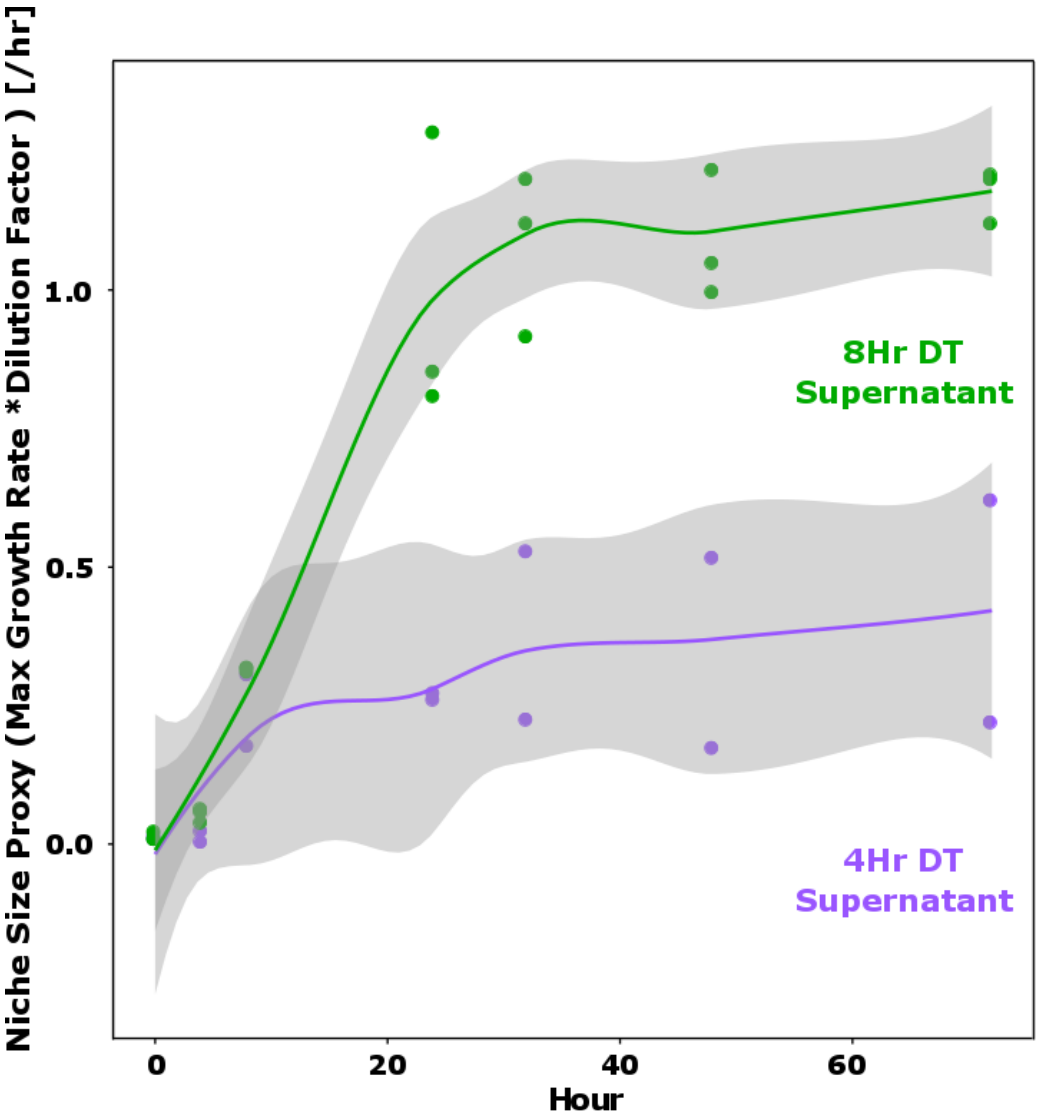

Figure 7 – The pool of organosulfur compounds differs in supernatants harvested from chemostats grown at differing dilution rates. The **[L-]** ancestor was grown under lysine limitation in a chemostat at one of two doubling times (8 Hr or 4Hr) to see if niche size would be affected (as a way to identify patterns for candidate molecule identification). Supernatants were harvested from chemostats from 0 to 72 hours and assayed for organosulfur by quantitating the growth rate of a constructed organosulfur auxotroph tester strain. Each dot represents a chemostat at given timepoint (5 chemostats total) while color represents doubling time of the chemostat. The curve and shading represent a general smooth fit of each set of conditions with 95% confidence intervals. The y-axis is a generic proxy for niche size (growth rate achieved by the tester strain multiplied by the dilution of the chemostat supernatant in the assay). According to this assay, there seemed to be a pool of organosulfur compounds that was 2-fold more concentrated in the 8 Hr doubling time chemostats relative to the 4 Hr doubling time chemostats.

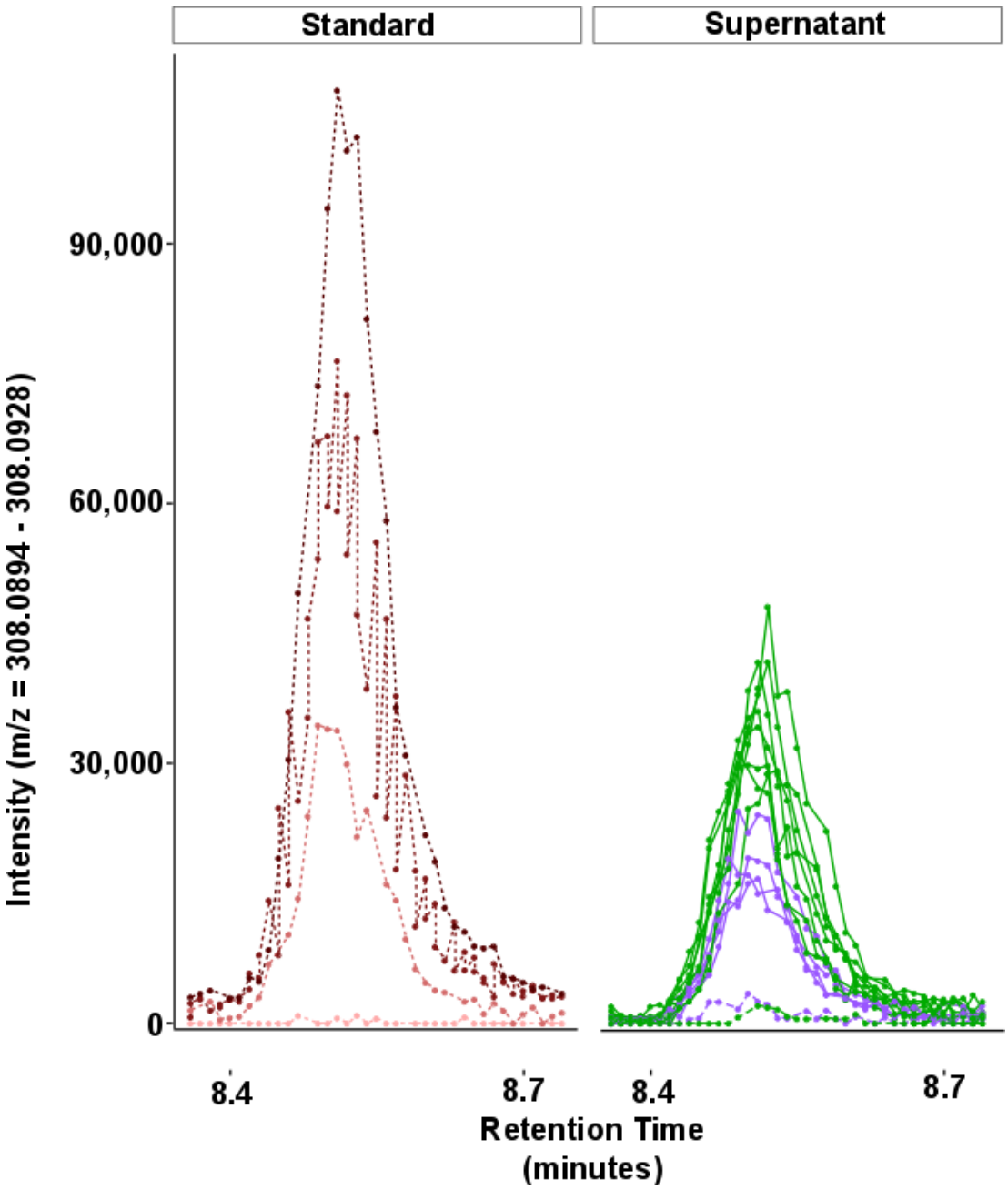

Figure 8 – Mass Spectrometry identifies glutathione present in chemostat supernatants. Supernatants harvested from lysine-limited chemostats one of two doubling times (8Hr or 4Hr) were subjected to untargeted mass spectrometry. Marker ion areas where screened to identify compounds that were higher in the 8Hr than the 4Hr doubling time. Left: 0 (dashed lines), 0.5, 1.0, and 1.5 µM reduced glutathione (GSH) in YNB+2% glucose run as standards. Right: Supernatants harvested at hour 0 (dashed lines), 48, and 72 from 8 Hr (Green) or 4 Hr (purple) doubling time lysine-limited chemostats. GSH appears to be 2-fold higher in the 8 Hr chemostats relative to the 4 Hour chemostats, consistent with the data in Fig.7.

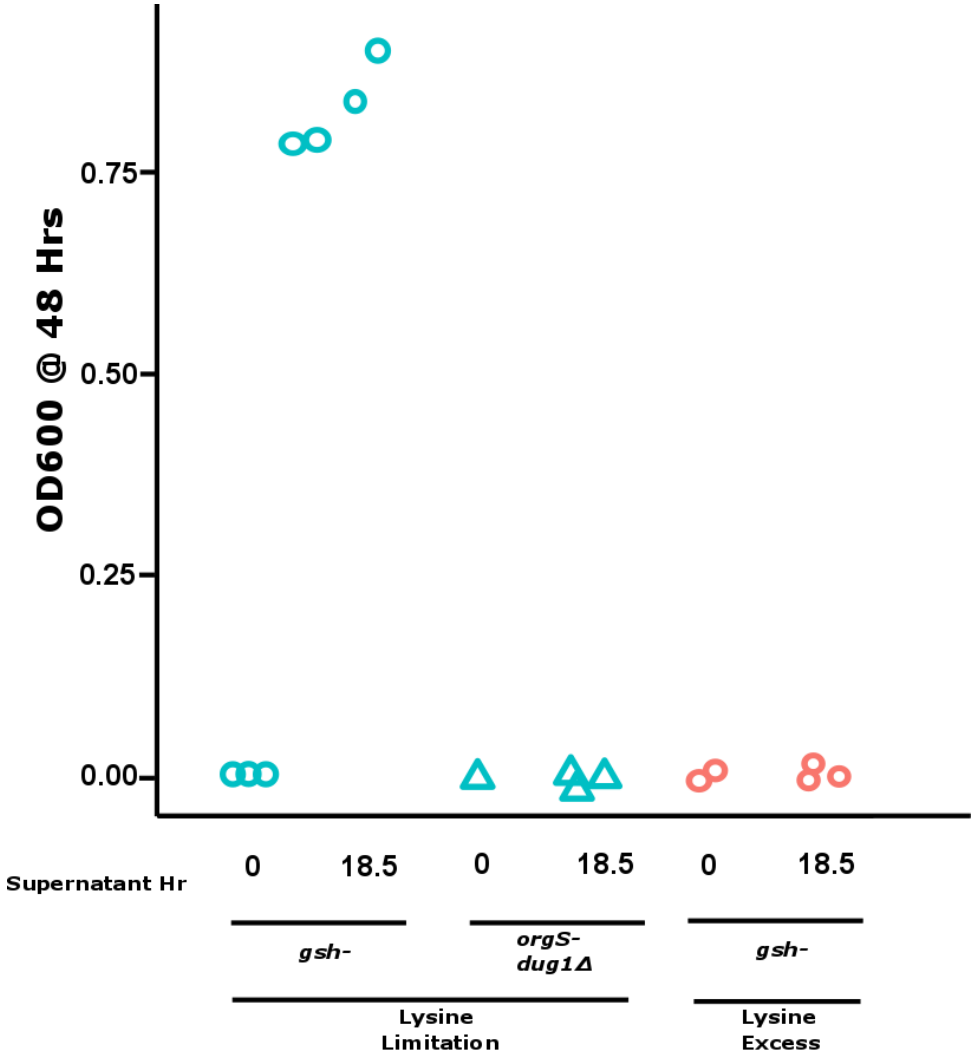

Figure 9 - Bioassays of chemostat and turbidostat supernatants indicates glutathione is the predominant niche and released in stress-dependent manner. WY2471 (*gsh1::KanMX*) and WY2473 (*met15 dug1::KanMX*) were grown to exponential phase in YNB+2% glucose and supplemented with 134 µM GSH or Met, washed, and starved. These strains were then inoculated into supernatants harvested from an 8 Hr chemostat (lysine limited, blue color) or a turbidostat (lysine excess, red color). Data are of final yield after 60 hours of growth. Growth of WY2471 in chemostat supernatant confirms the presence of glutathione, verifying the mass spectrometry data in Fig. 8.The failure of WY2473 to grow in chemostat supernatants suggest glutathione is a predominant compound. The failure of WY2471 to grow in turbidostat supernatants suggests the pool of GSH is much lower in turbidostats than chemostats, possibly through a combination of differing physiological release rates of GSH and differing dilution rates between (5-fold, 8Hr vs ~1.6 Hr).

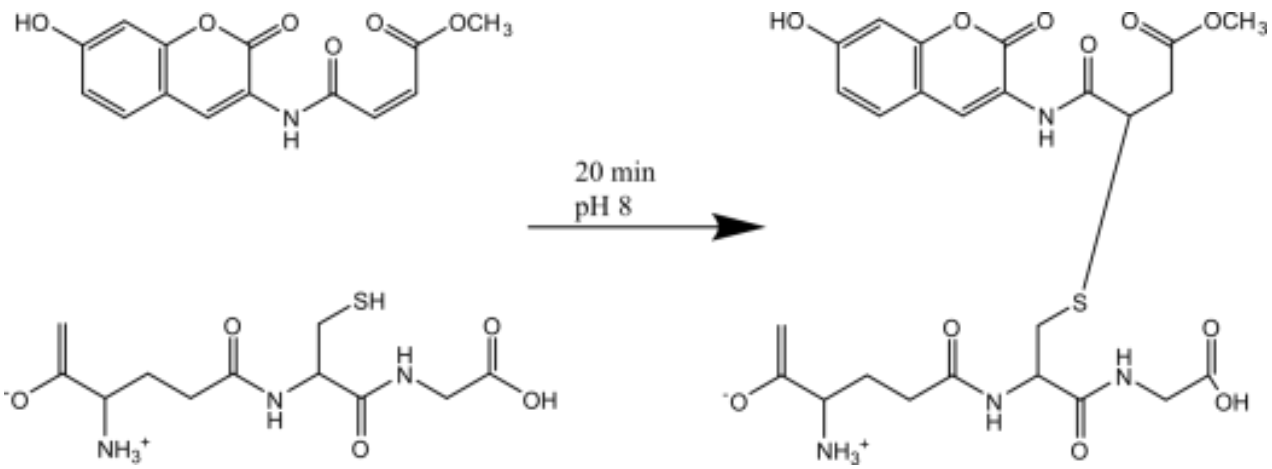

Figure 10 – A reaction to derivatize glutathione to a fluorescent compound for HPLC quantitation. Reaction diagram based on work by Yi *et al*[47].

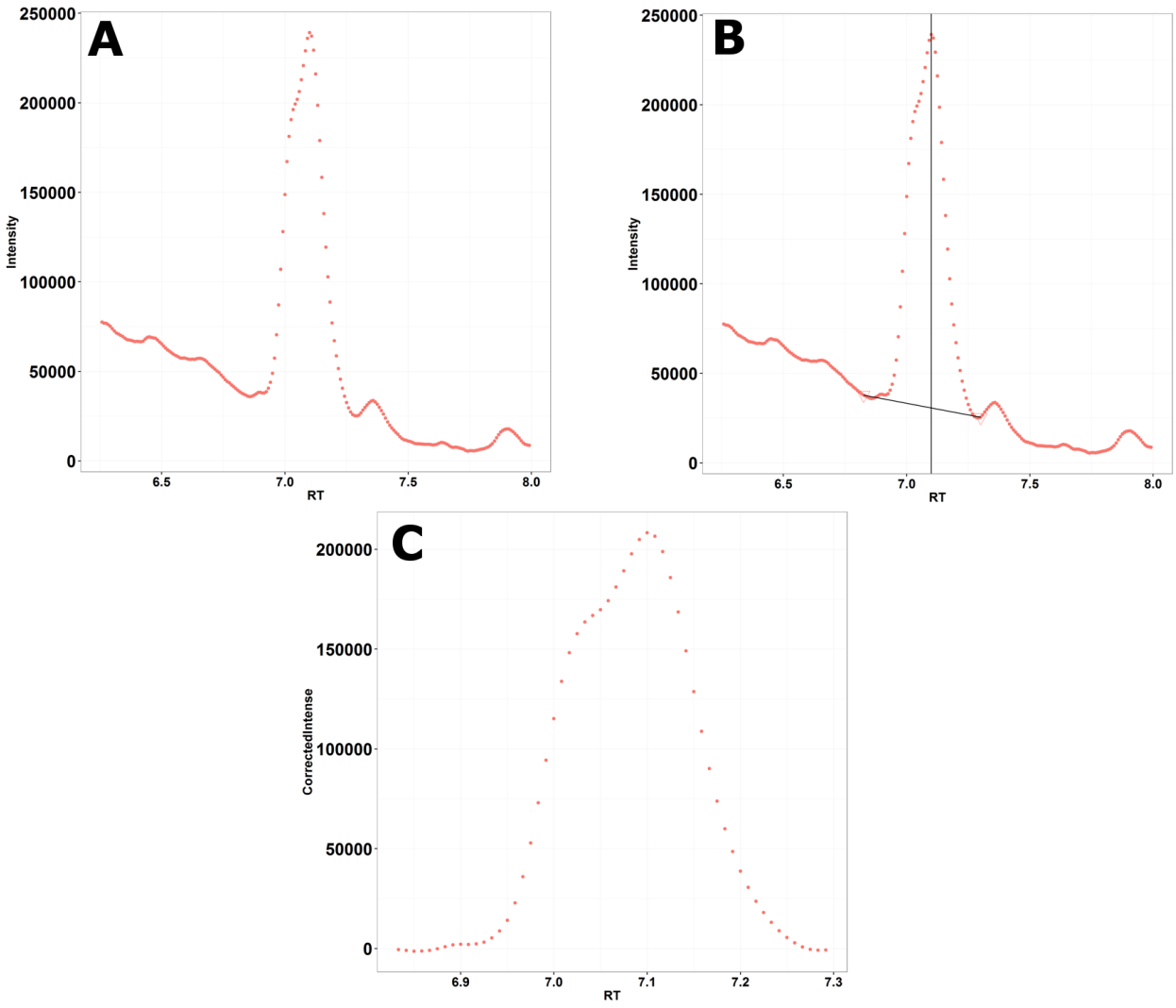

Figure 11 – Example of bioinformatics processing of glutathione HPLC data. All data were processed in the R environment. The y-axis corresponds to florescence intensity at 465 nm while the x-axis corresponds to retention time on the HPLC column. The raised signal at ~7 minutes corresponds to the reduced glutathione-probe conjugate eluting of the column and entering the florescence detector. A) Raw intensity (excitation at 400 nm and emission at 465 nm) for 0.5 µM GSH in YNB+2% glucose. B) Vertical line is the local maxima of data, while slope at baseline is the connection between two local minima on either side of local maxima (defined as the peak). C) Baseline correction and final output of R Script. The area of this curve is estimated using the trapezoidal method, which is then used for reduced glutathione quantification.

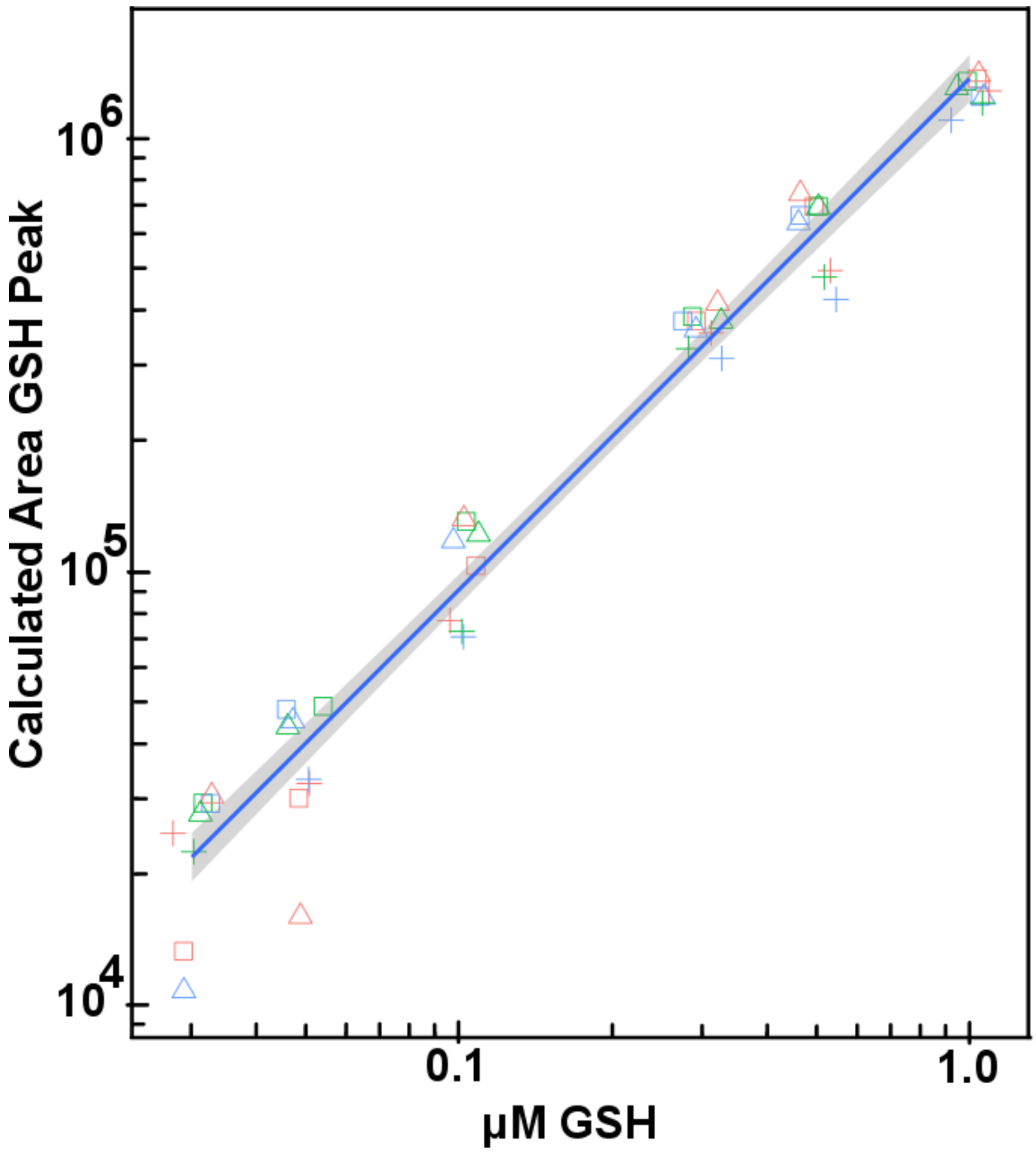

Figure 12 – A highly sensitive HPLC assay for quantitation of glutathione. Representative data of 3 independent standard curve (different colors), each measured 3 times (different shapes) in series.

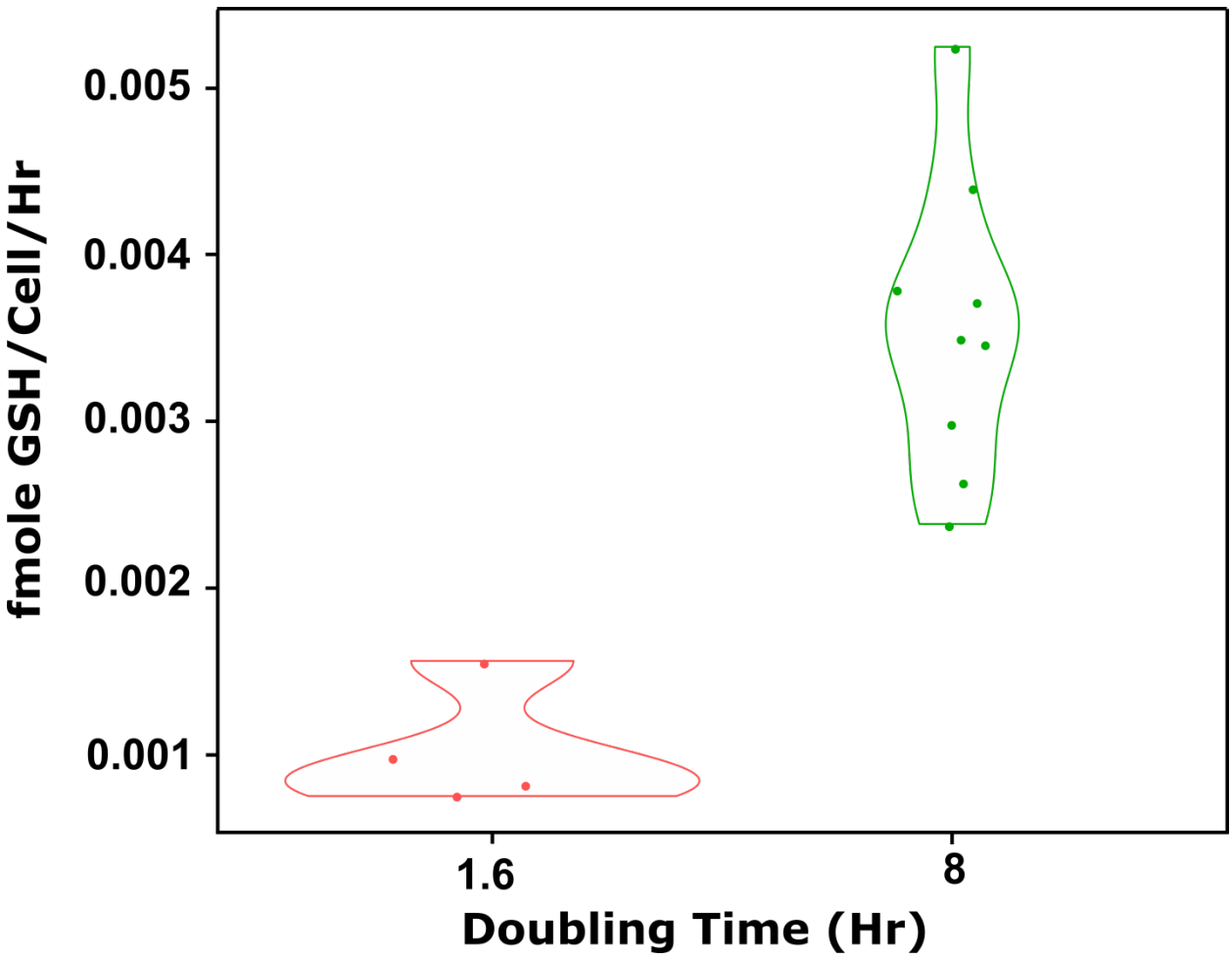

Figure 13 – Greater lysine limitation results in greater glutathione release rates. GSH levels were measured by HPLC and normalized by cell density (based on flow cytometry) of chemostat or turbidostat populations at the time of collection. Data were then multiplied by dilution rate (/Hr) to get release rates. Data assume steady state of chemostat/turbidostat populations. There is a ~2-fold difference in release rates of glutathione between lysine excess (1.6 hour doubling turbidostat) populations and lysine limited (8 hour doubling chemostat) populations.

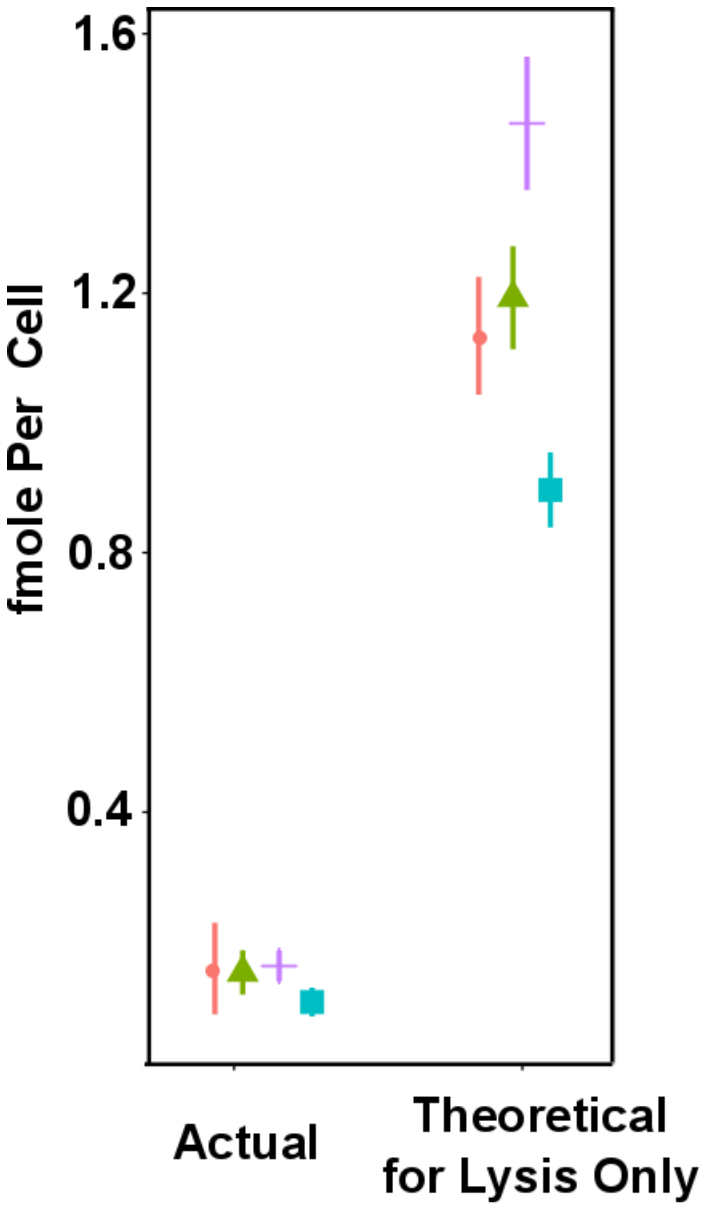

Figure 14 – Cell lysis cannot explain the total pool of reduced glutathione. Representative metabolite extraction data from 8Hr doubling chemostats. Chemostat populations were harvested and metabolism quenched by submersion into cold 40% Acetonitrile/ 40% methanol/ 20% water[44]. Metabolites were extracted and quantified by HPLC. This value was normalized by total number of cells harvested (quantified by flow cytometry). This number is how much glutathione on average could be attributed to a given cell. Next, chemostat supernatant GSH was quantified by HPLC and normalized by total number of dead cells. This value is how much each dead cell (determined by TO-PRO staining) would need to release in order for the total niche to be explained by cell lysis. Each shape corresponds to a unique chemostat. Error bars are 2 standard deviations of the GSH estimation by HPLC, normalized as described above.

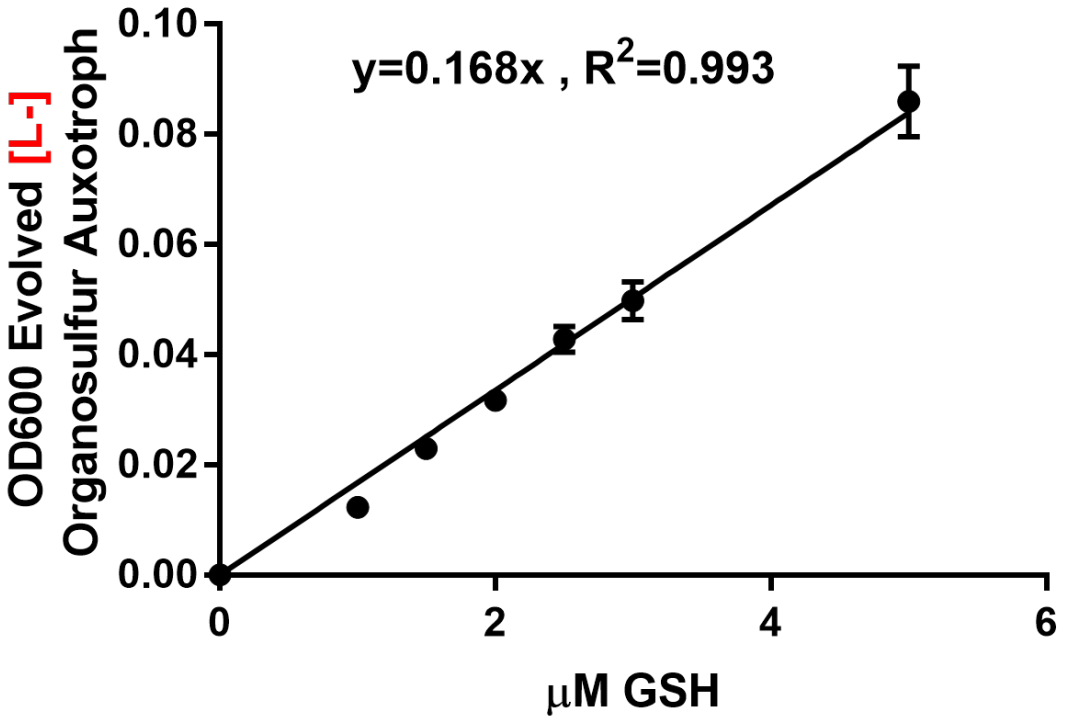

Figure 15 - Bioassay standard curve for quantifying total organosulfur niche size. Representative data of bioassay standard curve. WY1604 (evolved organosulfur auxotroph in **[L-]** background) was grown to exponential phase, starved for lysine, and used in a quantitative screen for organosulfur compounds. Data are of final yield after 60 hours of growth. Error bars correspond to 2 standard deviations based on 5 technical replicates per concentration of glutathione.

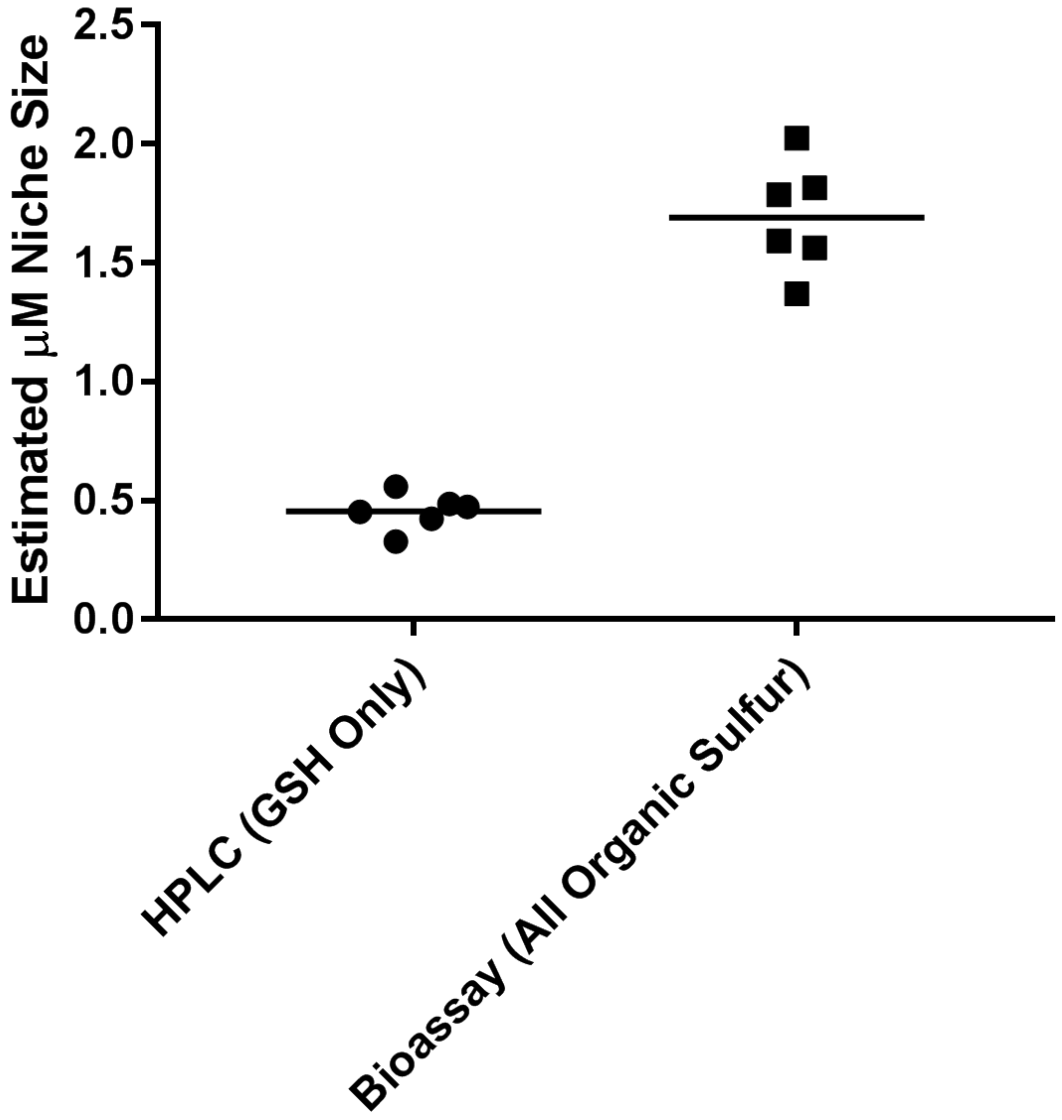

Figure 16 – Reduced Glutathione quantified by HPLC cannot explain the entire organosulfur niche. Representative data of comparing bioassay to HPLC. WY1604 (evolved organosulfur auxotroph in **[L-]** background) was grown to exponential phase, starved for lysine, and used in a quantitative assay for the organosulfur niche. Data are of final yield after 60 hours of growth. OD data were back-calculated to standard curve in Fig. 15. Data were compared against GSH quantification from HPLC. The 3-fold difference between these two assays suggest that reduced glutathione is roughly 1/3 of the total organosulfur niche.

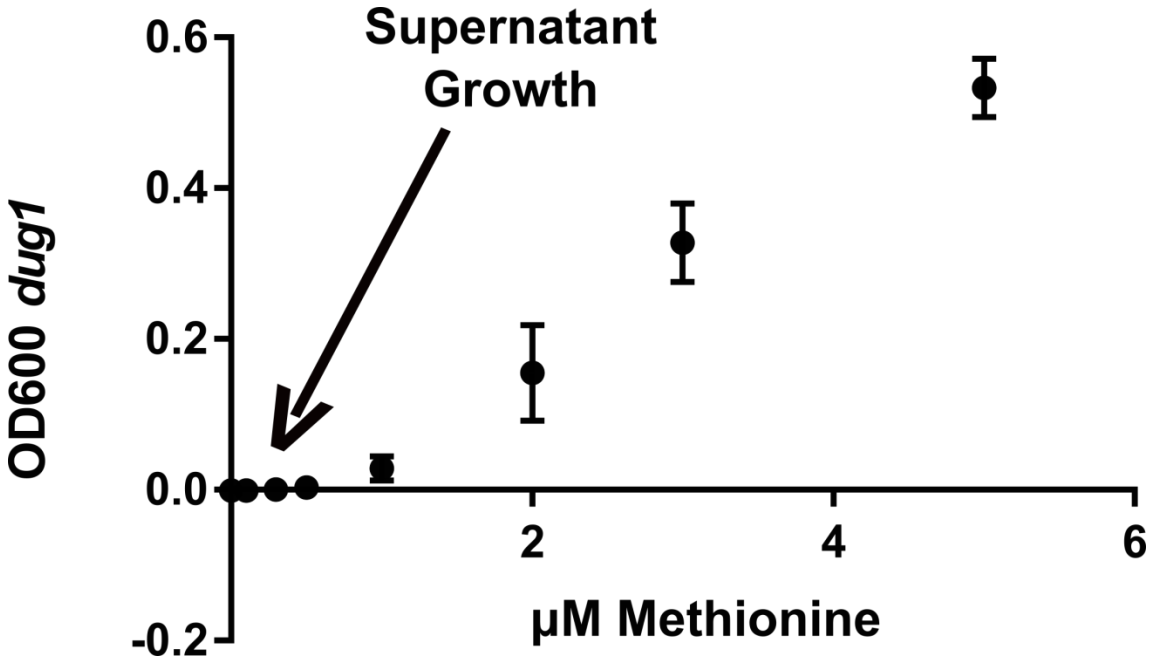

Figure 17 – *dug1* strain can grow at 1 µM Met, suggesting non-glutathione based organosulfur compounds in supernatant must be below this concentration. Representative data of bioassay standard curve. WY2473 (*met15 dug::KanMX*) were grown to exponential phase in YNB+2% glucose and supplemented with 134 µM Met, washed, and starved. This strains grows to detectable densities at 1 µM Met, but fails to grow in any supernatant tested. These data suggest that if any compound is not glutathione based, it must be below 1 µM methionine equivalent. Given that the difference between HPLC and bioassay results is also ~1 µM, these data suggest the entire organosulfur niche is glutathione based. Error bars indicate two standard deviations around the mean OD of the tester strain for each concentration of methionine.

Chapter 4. RESTORATION OF TOR SIGNIALING VIA ORGANOSULFUR AUXOTROPHY IS ADAPATIVE

4.1 ORGANOSULFUR AUXOTROPHY IS ADAPTIVE IN A NEGATIVE FREQUENCY-DEPENDENT MANNER

The next question I sought to address is how auxotrophic mutations could rise to the detectable frequencies in our study. I operated under the assumption that mutations can rise to high frequencies through one of four mechanisms: 1) genetic drift, 2) mutation-selection balance, 3) hitchhiking on highly beneficial backgrounds, and 4) *bona fide* competitive advantage. I ruled out genetic drift as a likely explanation early on, given that the population sizes from which the [L-] organosulfur auxotrophs were isolated from were on the order of a minimum of 10^6 cells for CoSMO batch culture and 10^8 cells for chemostat evolution runs. Additionally, the short evolutionary time ($\leq$150 generations for all lines) in which organosulfur auxotrophs arose in my experiments makes drift unlikely to explain the repeated emergence of auxotrophy. I also believe mutation-selection balance to be unlikely due to lack of evidence suggesting elevated mutation rate based on whole-genome sequence analysis (See Table 2).

This led to two likely hypotheses to explain repeated auxotroph emergence: hitchhiking with highly beneficial alleles or actual competitive advantage. Hitchhiking in rapidly evolving populations has previously been observed with both yeast[64] and *E. coli*[65]. Additionally, previous work has demonstrated that in the **[L-]** background, highly adaptive mutations can sweep through populations rapidly[24] from the beginning of an experiment, making it reasonable to think that a neutral or slightly deleterious auxotrophy-causing mutations could have risen to the

frequencies observed in our study, provided that the required niche was being generated as our data indicate.

To resolve these two possibilities, I decided to test the relative fitness of a **[L-]** organosulfur auxotroph against an otherwise isogenic **[L-]** organosulfur prototroph at differing starting ratios in a lysine-limited environment. I reasoned that if organosulfur auxotrophy was beneficial under these conditions, this benefit would be a *negative frequency-dependent* advantage. If auxotrophy was truly beneficial under these conditions, this adaptive benefit should diminish as organosulfur auxotrophy became more common in the **[L-]** population, since increase in organosulfur auxotroph frequency will result in decrease prototroph frequency, thus diminishing the size of the niche required to support auxotrophs. If organosulfur auxotrophy had risen to high frequencies from hitchhiking with highly adaptive mutations, then I would expect auxotrophy to carry no detectable benefit or possibly a fitness deficit in our competitions even if **[L-]** organosulfur auxotrophs were rare in the population with ample access to the required niche.

Replicate populations **[L-]** organosulfur auxotrophs and **[L-]** organosulfur prototrophs were inoculated at various starting ratios and population dynamics were tracked for ~15 generations by flow cytometry. I observed dynamics consistent with **[L-]** organosulfur auxotrophs having a negative frequency-dependent advantage over **[L-]** organosulfur prototrophs (Fig. 18). When inoculated at a ratio below 0.1 (~10% of the population), **[L-]** organosulfur auxotrophs quickly rose in frequency within the first 10 generations before being maintained about a ratio of ~0.1. When inoculated at ratios near 0.1, **[L-]** organosulfur auxotrophs were qualitatively retained around this ratio for the 15 generations I tested. If inoculated at ratios

greater than 0.1, **[L-]** organosulfur auxotrophs quickly dropped in frequency within the first 10 generations before stabilizing around a ratio of 0.1.

From these data I conclude that organosulfur auxotrophs confers a fitness advantage when its niche has been provided by organosulfur prototrophs. These auxotrophs can rise to high frequencies due to this adaptive advantage, but only to the degree that they are still supported by the prototrophic population, existing in negative frequency-dependent relationship.

4.2 The Selective Advantage of Organosulfur Auxotrophy Cannot be Explained By Energy Savings

A common argument for the observed fitness benefit of auxotrophic strains over their prototrophic counterparts is known as the 'energy-saving hypothesis'. Briefly, strains have an energy budget that must be optimized for growth. Auxotrophic strains can save energy by not making the metabolite being provided to them. For example, strains with loss of function mutations in a biosynthetic pathway should save on the energy costs of running the biosynthetic pathway. Theoretically, auxotrophs with full gene deletions should save additional energy by not translating the deleted gene. As a result, auxotrophs should have a fitness benefit in the form of more efficient growth (higher birth rate / greater fecundity).

This hypothesis is popular in the literature, particularly in the field of modeling[66]. Additionally, experimental evidences based on synthetically constructed auxotrophs support this[50, 67]. However, the hypothesis is not universally accepted[66,68]. I wanted to address this in our system. In the moderately constant environments of nutrient limitation, such as a chemostat or cross-feeding co-culture, I reasoned that fitness gains could be explained predominantly by increasing the birth rate (such as increasing affinity to limiting metabolites [69]) or decreasing the death rate of a cell. Therefore, an ideal assay would be one where birth and death could be

quantified separately. To do this, I developed a high-throughput microscopy-based assay (See Materials and Methods) where I were could quantify the two aspects of net growth rate (birth rate and death rate). As a first step, I grew otherwise isogenic organosulfur auxotrophs and prototrophs in concentrations of lysine and glutathione where both are provided in excess according to Monod kinetics. I reasoned that if organosulfur auxotrophs were saving energy, then their birth rates under excess nutrient conditions should be higher than the organosulfur prototrophs. In this assay I assumed that the death rate is negligible over the duration of the experiment since the required nutrients are provided in excess, which is supported by data generated from our lab (not shown here). Surprisingly, I observe that the organosulfur auxotrophs grow significantly slower (by nearly 30%) under nutrient excess conditions [70](Fig. 20). I therefore concluded that the fitness benefit of these auxotrophs cannot be explained by the energy savings hypothesis.

4.3 Organosulfur Auxotrophs are More Viable under Lysine Limitation than Prototrophs

I reasoned that since there was no obvious gain in cell fecundity, fitness benefit of organosulfur auxotrophy must be explained by enhanced viability. It is known that lysine auxotrophs when starved for lysine undergo rapid death [14]. Additionally, organosulfur auxotrophy has previously been shown to be different compared to other yeast autotrophies – when yeast are made auxotrophic for organosulfur, they actually show viability *similar* to yeast starved for 'natural' nutrients such as glucose or sulfate[59,71]. Therefore, I hypothesized that organosulfur auxotrophs could have better viability (a slower death rate) under starvation for lysine, provided that organosulfur was also limiting. Theoretically, the enhanced viability of organosulfur auxotrophy would be removed if organosulfur was then provided in excess, since

the burden of lysine auxotrophy would not be compensated for. To test this, I studied the death kinetics of an organosulfur auxotroph and an otherwise isogenic organosulfur prototroph under conditions of no lysine both with and without glutathione present (see Fig. 21).

All strains showed the predicted viability dynamics. Organosulfur prototrophs (blue circles) in the **[L-]** background quickly die in the absence of lysine (two left panels) regardless of glutathione being present. Organosulfur auxotrophs (yellow circles) showed little to no death (gradual increase in florescence corresponds to residual growth) under conditions of no glutathione, but rapidly die when glutathione is supplied in excess. Indeed, organosulfur auxotrophs supplied with glutathione look exactly like organosulfur prototrophs in terms of death dynamics. Ultimately, the enhanced viability that an organosulfur auxotroph likely gains under competition would likely be due to a lower probability of death under restriction for lysine, provided that glutathione (or any other possible organosulfur molecule in the niche) are limiting as well.

4.4 Rapamycin can rescue the Death of Lysine Auxotrophs under Lysine Limitation

A common explanation for the rapid death of **[L-]** under lysine limitation is that certain yeast auxotrophies have an 'unnatural' physiological response to limitation for the required metabolite. While starvation for a 'natural' nutrient, such as glucose, elicits distinct physiological responses meant to prolong survival[4, 12, 7], many yeast auxotrophs starved for their required metabolite have a markedly different response in gene expression[14]. Many 'wasteful processes' occur, such as continued fermentation and amino acid synthesis, which results in a cellular 'burning out' and rapid death. Lysine auxotrophs show this characteristic pattern[14]. One mechanistic explanation is the processing of information for different nutrients is wired

differently. While primary metabolites like glucose are sensed through the TOR pathway (specifically using the TORC1 complex[16]), certain amino acids like lysine are not[14]. In this media (YNB+2% glucose), TORC1 is turned on due to the high concentrations of natural nutrients such as glucose and phosphate, and because the cell doesn't 'think' it's limited for any metabolites, it proceeds to a wasteful fashion despite not having essential lysine available. This results in rapid death.

If this model is correct, then treating the **[L-]** organosulfur prototrophic strain with rapamycin, which targets and specifically inhibits TORC1[72, 73], should rescue the rapid death phenotype. This was indeed the case. Fig. 21 (top right panel) shows death kinetics of the evolved **[L-]** organosulfur prototrophic strain under lysine limitation with 1 µM rapamycin, which is consistent with previous studies in yeast[71,4,74]. The **[L-]** organosulfur prototroph now match the **[L-]** organosulfur auxotrophs death profile – little to no death.

Treatment with rapamycin also rescues the death of **[L-]** organosulfur auxotroph in excess glutathione (lower right panel). This is also expected based on the current model – organosulfur (specifically methionine) has been shown to be sensed by the TORC1 complex via methylation of PP2A mediated by Ppm1[19]. Therefore, absence of organosulfur for an organosulfur auxotroph would result in TORC1 being turned off, while supplying organosulfur would result in TORC1 being turned on, which in the absence of lysine would result in death. Rapamycin can override TORC1 being turned on by the presence of glutathione, thus allowing organosulfur auxotrophs to survive in the absence of lysine but in excess glutathione. Taken together, this data suggests that the TOR pathway, specifically the rapamycin-sensitive TORC1, is important in understanding the enhanced viability of organosulfur auxotrophs under limitation for both glutathione and lysine.

4.5 Abolishing Autophagy, a Downstream Effector of TOR, Partially Removes the Survival Phenotype

If TORC1 was important, than removing a process important to survival when TORC1 is turned off should abolish the enhanced viability of the organosulfur auxotrophs. An important downstream process of TORC1 is autophagy, which is the process of cellular self-digestion in response to limitation for required metabolites[75, 76]. Under normal conditions when TORC1 is turned on (in the presence of plentiful nutrients), Atg13 is phosphorylated and exists as a monomer. When TORC1 is turned off, Atg13 forms a complex with Atg17 and the kinase Atg1 to form the autophagy initiation complex[77, 78]. Following this, a complicated, multifaceted process occurs where cellular vesicles called 'autophagosomes' are formed and expanded, encompassing cellular machinery for degradation and traffic to the vacuole for degradation (for a complete review of known processes/parts involved in autophagy in yeast, see [78]). Autophagy requires at least 17 separate proteins for proper function, including Atg5, which is required for autophagosome formation[79]. Knocking out *ATG5* in yeast has been shown to abolish autophagy[71].

I reasoned that if TORC1 was important in the survival of the **[L-]** organosulfur auxotrophs, then abolishing autophagy (a known downstream process) should at least diminish the survival observed under limitation for lysine and glutathione. To test this, I knocked out *ATG5* in the **[L-]** organosulfur auxotroph and subjected it to the same panel of tests outlined above (Fig. 21, green circles). Knocking out *ATG5* in the organosulfur auxotroph seems to abolish the survival; regardless of the presence of glutathione or rapamycin (all panels show roughly the same death kinetics).

These data suggest that autophagy is required for the enhanced viability of organosulfur auxotrophs, further indicating that TORC1 signaling is an important player in the observed fitness benefit.

4.6 The Advantage of Organosulfur Auxotrophy is Facilitated by the Reacquisition of TOR Signaling

In this chapter I have addressed how organosulfur auxotrophs rose to high frequencies in my **[L-]** lineages once the required organosulfur niche is present. In chapter 3, I demonstrated that lysine limitation drives live **[L-]** cells to release glutathione, providing the niche to support auxotroph evolution. Organosulfur auxotrophy carries an adaptive benefit and can exist in a negative frequency-dependent manner with organosulfur prototrophs. This dynamic makes intuitive sense. The organosulfur prototrophs provide the glutathione that the auxotrophs require – therefore they can only rise to a frequency such that they are still supported by the prototrophic population[80]. This new interaction is theoretically stable, assuming that neither player gains a mutation that skews the fitness benefit.

The fitness benefit of a mutation in a relatively constant environment can be parsed into two categories: mutations that affect the fecundity of a cell and ones that affect the viability of the cell. Both factors could contribute to a higher max growth rate/fitness (higher fecundity or higher viability/lower death rate). A popular hypothesis in the literature to explain auxotroph ubiquity in microbial communities and in evolutionary models is that auxotrophs save energy by not making metabolites that they can scavenge from the environment [50,67,81]. However, this hypothesis is not universally accepted. Dykuizen and Stoebel *et al*. argued that the energy savings argument is too simplistic given the complex nature of metabolic regulation[66,68]. Additionally, adaptive benefits of gene deletions have been shown to have higher pleiotropic

effects, particularly related to redistribution of metabolic fluxes[61]. Therefore, I designed a high-throughput assay to assess both viability and fecundity simultaneously. I reasoned that if the organosulfur auxotrophs were saving energy, then that would translate to an enhanced growth rate under excess lysine and glutathione conditions. This was not the case, as organosulfur auxotrophs grew ~30% slower even under nutrient excess conditions.

Given that enhanced fecundity/energy savings could not explain the adaptive benefit of auxotrophs, I then studied their viability under starvation for lysine and glutathione. I found that organosulfur auxotrophs were more viable than organosulfur prototrophs, provided that glutathione was limiting. These data were suggestive of the role of TORC1 in mediating this phenotype. TORC1 is suspected to be bypassed by lysine auxotrophs in yeast[14] while organosulfur is known to be sensed (through other mediators) by TORC1 [19], so starvation for both metabolites would result in different physiological states, which has also been previously characterized[59,59]. Additionally, treatment with rapamycin rescues the poor viability of the **[L-]** organosulfur prototrophs when they are starved for lysine, which is again suggestive of lysine auxotrophs bypassing TOR regulation. Finally, abolishing autophagy, a known downstream process of TORC1[77], by knocking out *ATG5* diminishes the survival of organosulfur auxotrophs regardless of whether TORC1 is off or on.

These data taken together suggest that the adaptive benefit of organosulfur auxotrophy is mediated via the reacquisition of TOR signaling in response to nutrient limitation. By becoming auxotrophic for organosulfur, these strains now can lower their death rate under limitation for lysine, provided that glutathione is also limited. This creates a scenario where if two cells, an organosulfur auxotroph and an organosulfur prototroph, both are competing for nutrients but none are readily available, than the organosulfur auxotroph has a lower probability of dying

compared the organosulfur prototroph, which still has the misregulated metabolism. This scenario, over time, would generate a situation where on average organosulfur auxotrophs die slower than organosulfur prototrophs, thus increasing in frequency.

The above point is a profound example of how evolution can generate novel and non-intuitive solutions to problems. A simpler type of adaptation to the misregulated TOR metabolism could be selection for a suppressor phenotype that doesn't result in an auxotrophic requirement. Indeed, loss of function mutations in *PPM1*, a methyltransferase involved in TORC1 regulation (and also used in TORC1 sensing methionine[19]) have been shown to mitigated the rapid death of leucine auxotrophs starved for leucine, which also bypass TOR similar to lysine[4]. However, I see no evidence of any mutation in *PPM1* in any of the sequenced clones in this study (See Table S2) or any other sequenced clones from similar evolution experiments from this lab (data not shown). However, a similar phenotype could be achieved by becoming auxotrophic for organosulfur, which was facilitated by the ecological processes being driven by the lysine limitation, as outlined in chapter 2. Ultimately, the organosulfur auxotrophs could arise because there is a niche to support them, thus allowing mutations that previously would have been too deleterious to establish an evolutionary opportunity for success.

4.7 Materials and Methods

Strain Construction

All strains are used in this chapter listed in Table S1.A evolved organosulfur auxotroph (WY1604) was used as the progenitor strain for making relevant strains in this chapter. First, the *fba1::FBA1-mCherry-loxp* allele was replaced by *fba1::FBA1-BFP-KanMX* allele by lithium acetate transformation [82]. Strains were selected on YPD supplemented with kanamycin and

integration was confirmed by checking for BFP fluorescence by flow cytometry. This created the strains WY2072/WY2073. Next, the wild-type allele of *MET10* was amplified from WY1335 (the ancestral **[L-]** strain) and transformed into WY1604 to generate at *met10 ::MET10* evolved **[L-]** mCherry strain using a standard lithium acetate protocol[82]. Selection was performed by plating on YNB+2% Glucose + 164 µM lysine (media lacking methionine). This generated strain WY2429. WY2370, which was used to test the effects of abolishing autophagy by knocking out *ATG5,* was constructed amplifying at Nourseothricin resistance cassette from plasmid DNA, gel purified, and transformed into WY1604 using a standard lithium acetate procedure [82]. Section was performed on YPD plates supplemented with nourseothricin and integration at the *ATG5* locus was confirmed by PCR and Sanger sequencing. All transformants were screened for obvious growth defects by measuring growth in YND+2% Glucose and any additional supplements required. Strains were also frozen in 15% glycerol for future use at -80 C.

Competition Experiments

To facilitate experiments where I could perform multiple replicates, I chose to use the coculture system of CoSMO to mimic the lysine limited environment, reasoning that due to the parallel nature of mutations found in both coculture and monoculture lysine limited chemostats meant the environments were similar. To generate a CoSMO environment for competition, WY1340 (the purine requiring/lysine overreleasing strain in the RM11 background) was grown to exponential phase overnight in YNB+2%glucose+134 µM adenine, washed 3 times with YNB+2% glucose to remove excess adenine, and starved for 24 hours to totally deplete vacuolar storage. During this starvation, WY2702/2073 (the BFP *met10* evolved clones) and WY2429 (the mCherry *MET10* evolved clone) was grown overnight in YNB+2% glucose + 134 µM methionine to exponential phase, washed 3 times, washed 3 times with YNB+2% glucose to

remove excess methionine and lysine, and concentrated into YNB+2% glucose. Next, WY2072/3 and WY2429 were mixed in ratios of 1:100, 1:10, 1:1, and 10:1 to a final OD600 of 0.1. This mixture of populations was then added 1:1 with WY1340 to a final OD600 of 0.03. This was considered generation 0. Populations were monitored for growth by measuring optical density over time and periodically diluted back to OD600 0.03 (OD600 was never greater than 0.45 to ensure no additional metabolites from YNB +2% glucose was limiting). The OD600 data was used to back calculate total generations in the experiment. Periodically, 100 μL of the culture was saved for flow cytometry to track mCherry and BFP ratios. Experiments were performed until the BFP:mCherry ratio stabilized around 0.1.

Microscopy Experiments

The methodology for these experiments follows the same procedure as outlined in Chapter 3 with a few differences. The strains tested (fully detailed in Table S1) were WY1604 (evolved mCherry *met10*), WY2429 (evolved mCherry *met10::MET10* derived from WY1604*)*, and WY2370 (evolved mCherry *met10 ATG5::NAT* derived from WY1604). In each well of a 96 well plate, one of these strains was inoculated into a known amount of YNB+2% glucose supplemented with a known amount of lysine, glutathione, and rapamycin. Figure 20 showing growth rate data is in the presence of 20 μM lysine. Figure 21 showing differing death dynamics contains no lysine (0 μM). In cases where death rate was assessed, normalized fluorescence intensity was plotted against time for the entire experiment. A decrease in fluorescence intensity was been shown by our group to correlate well with cell death (data not shown).

4.8 FIGURES

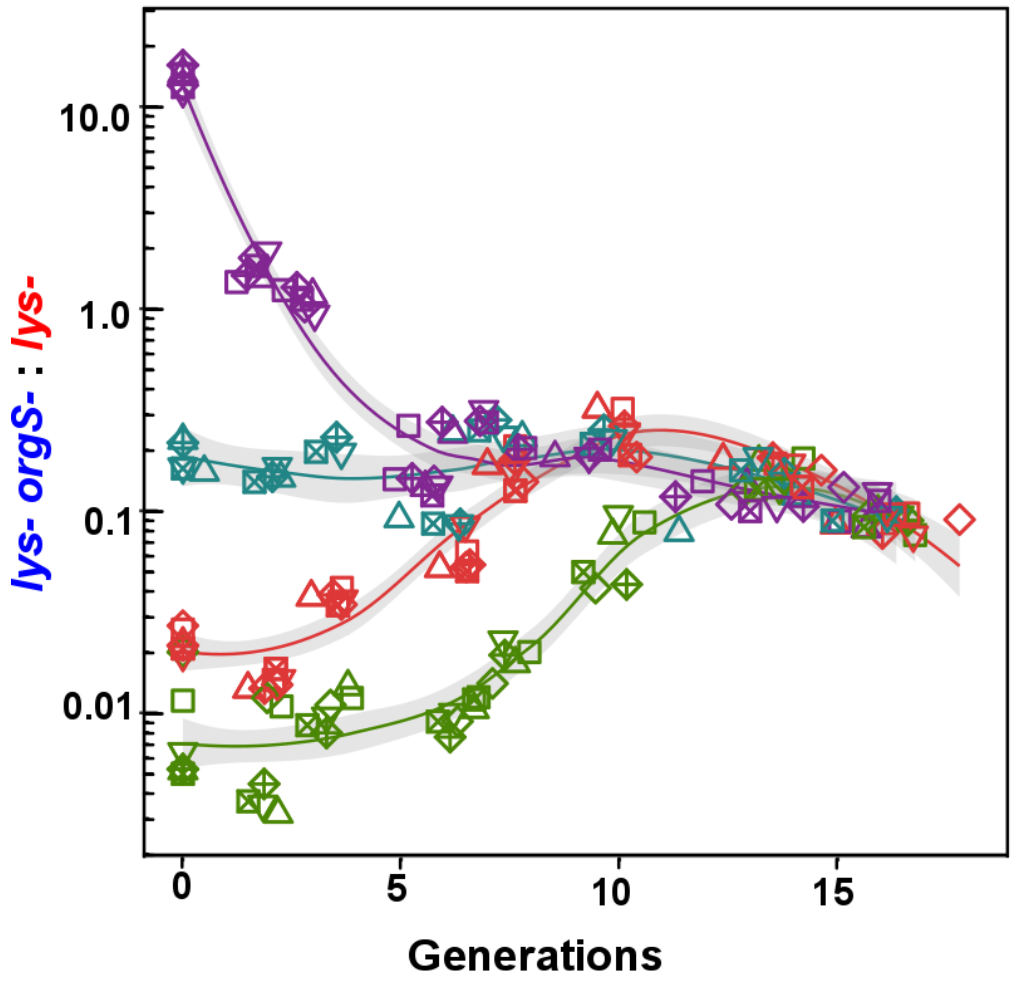

Figure 18 – Organosulfur Auxotrophy is beneficial in a negative frequency-dependent manner. Representative competition data of a lysine-limited environment via CoSMO. BFP-tagged evolved organosulfur auxotrophs and otherwise isogenic mCherry organosulfur prototrophs (see Table 1 for all strains), were grown to exponential phase in YNB+2% glucose+164 µM lysine +134 µM Met, washed free of metabolites with YNB+2% glucose, and inoculated at various ratios to 50% of a culture, with the other 50% consisting of a GFP lysine-releasing strain (WY1340, part of CoSMO). This was done to facilitate competition experiments. Colors were tracked over time by flow cytometry. Competition dynamics between organosulfur auxotrophs and prototrophs is suggestive of negative frequency-dependent dynamics.

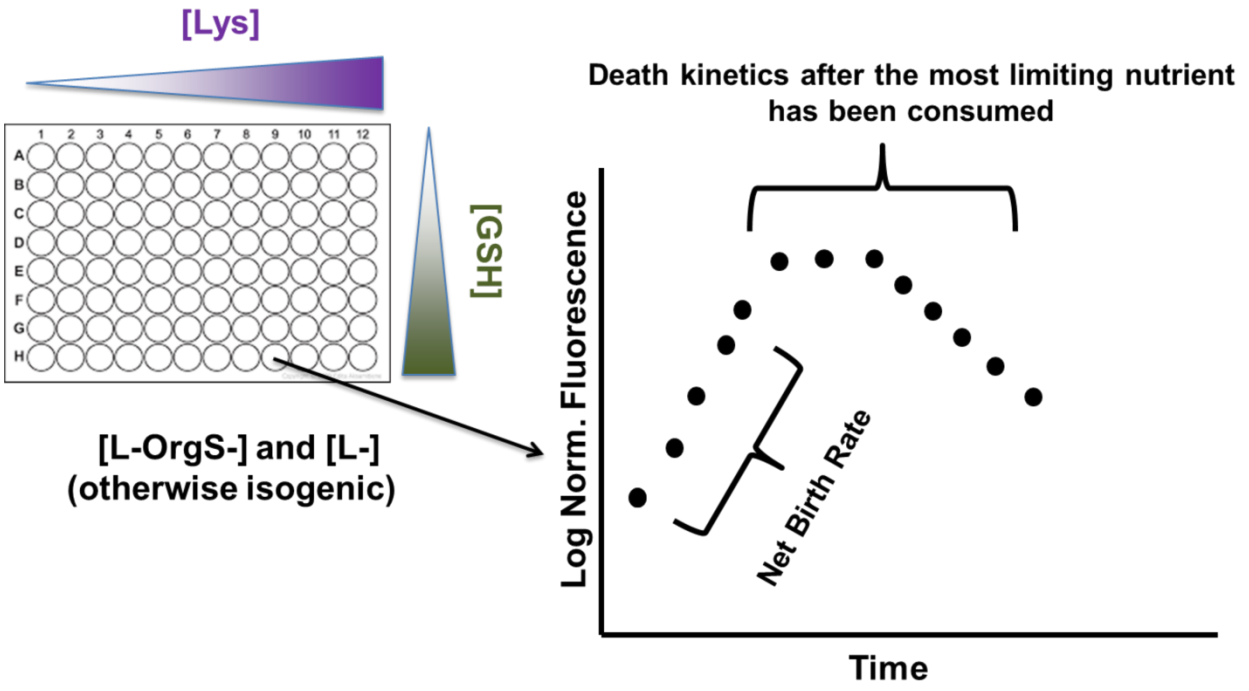

Figure 19 – A high-throughput assay for the assessment of viability and fecundity. Strains were grown in YNB+2% glucose+164 µM lysine + 134 µM GSH, washed 3 times in YNB+2% glucose, and starved for at least 3 hours to deplete vacuolar stores. 1,000-10,000 cells were then inoculated into a well of YNB+2% glucose plus known amounts of lysine, glutathione, and with or without rapamycin and imaged on a robot microscope. All strains were mCherry tagged (see Table 1). Increase in fluorescence intensity correlates with cell growth while decrease in fluorescence intensity correlates with cell death.

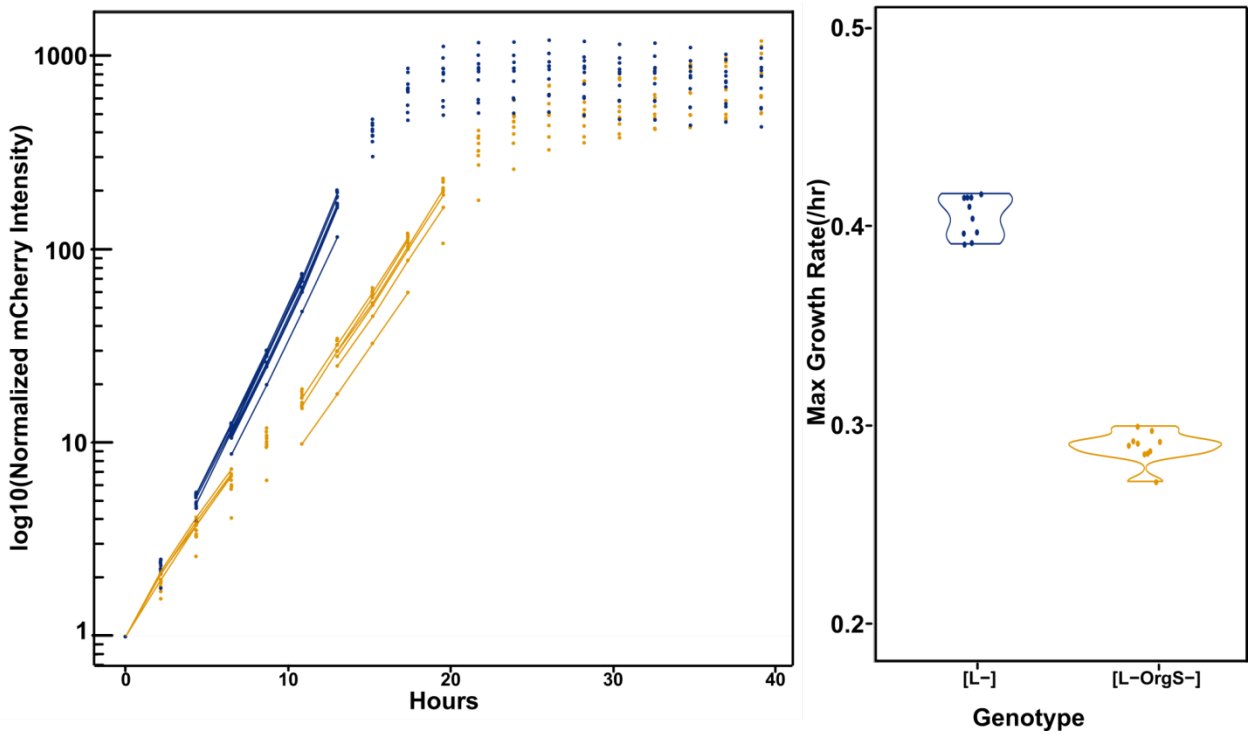

Figure 20 – The selective advantage of organosulfur auxotrophy cannot be explained by energy savings. Representative data of WY1604 (yellow, evolved organosulfur auxotroph) and WY2429 (blue, isogenic organosulfur prototrophs derived from WY1604) grown in 20uM lysine and 20 µM GSH. Right: Intensity dynamics against time. Solid lines correspond to 4 slice/~8hour window where max growth rate is calculated. Left: Violin plots of max growth rates for each strain. The 30% difference in growth rate between WY1604 and WY2429 suggests that the adaptive benefit of organosulfur auxotrophy cannot be explained by energy savings/more efficient growth under nutrient availability.

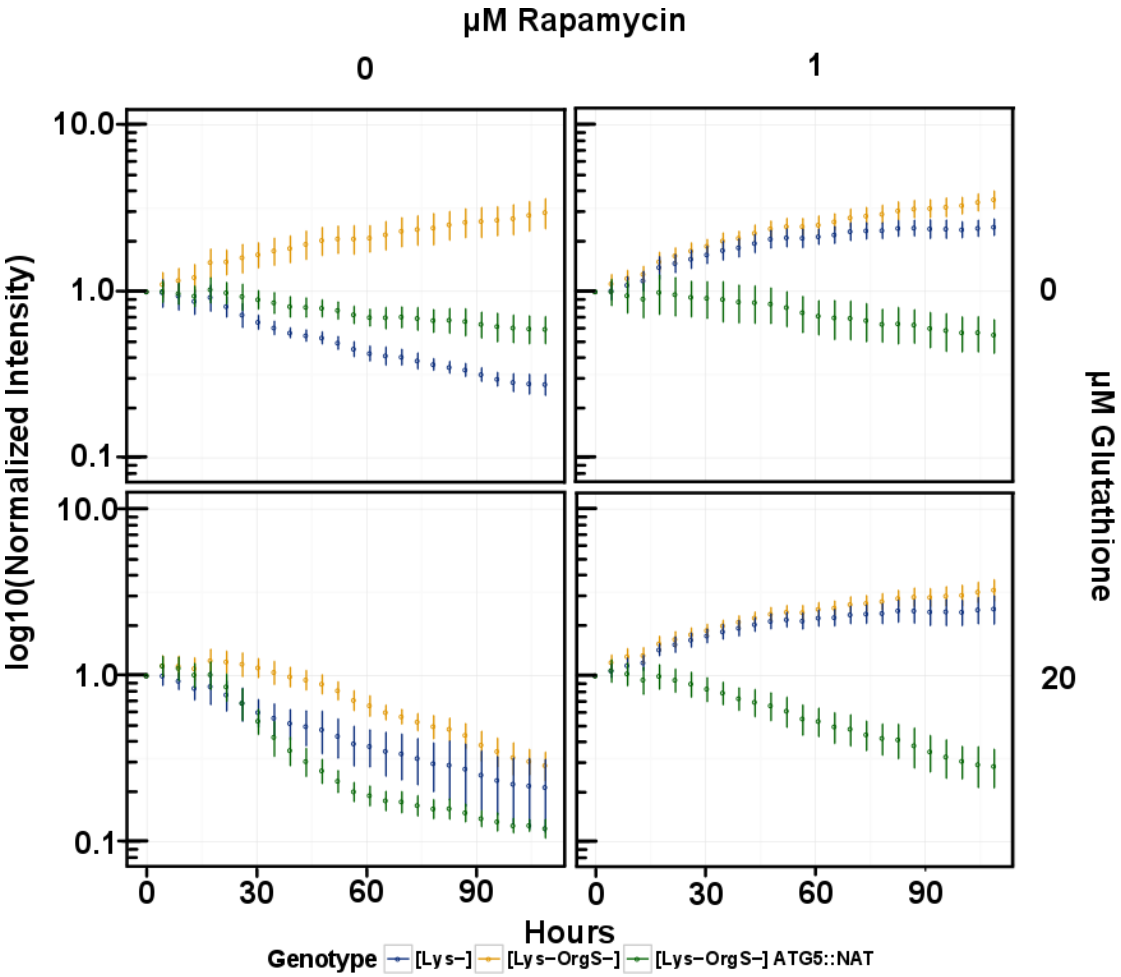

Figure 21 – Viability Dynamics of Organosulfur auxotrophs and prototrophs in an otherwise isogenic evolved [L-] background. Top Left: dynamics in YNB+2% glucose (no GSH, no rapamycin). Bottom Left: dynamics in YNB+2% glucose+ 20 µM GSH (no rapamycin). Top Right: dynamics in YNB+2% glucose+1 µM rapamycin (no GSH). Bottom right: dynamics in YNB+2% glucose+20 µM GSH +1 µM rapamycin. Yellow corresponds to WY1604 (evolved organosulfur auxotroph). Blue corresponds to WY2429 (isogenic organosulfur prototrophs derived from WY1604), green corresponds to WY2370 (evolved organosulfur auxotroph with *ATG5* knocked out, derived from WY1604). WY1604 shows enhanced viability relative to WY2429 in the presence of no lysine and no glutathione (top right). Adding glutathione removes this viability (bottom left). Adding rapamycin rescues the poor viability of WY2429 in the absence of lysine (top right), and in the presence of GSH for WY1604 (bottom right). Abolishing autophagy greatly diminishes the viability advantage of WY1604 under limitation for GSH. All data are consistent with WY1604 regaining TORC1 regulation compared to WY2429. Error bars correspond to 2 standard deviations of average normalized mCherry intensity for 5 replicates/wells.

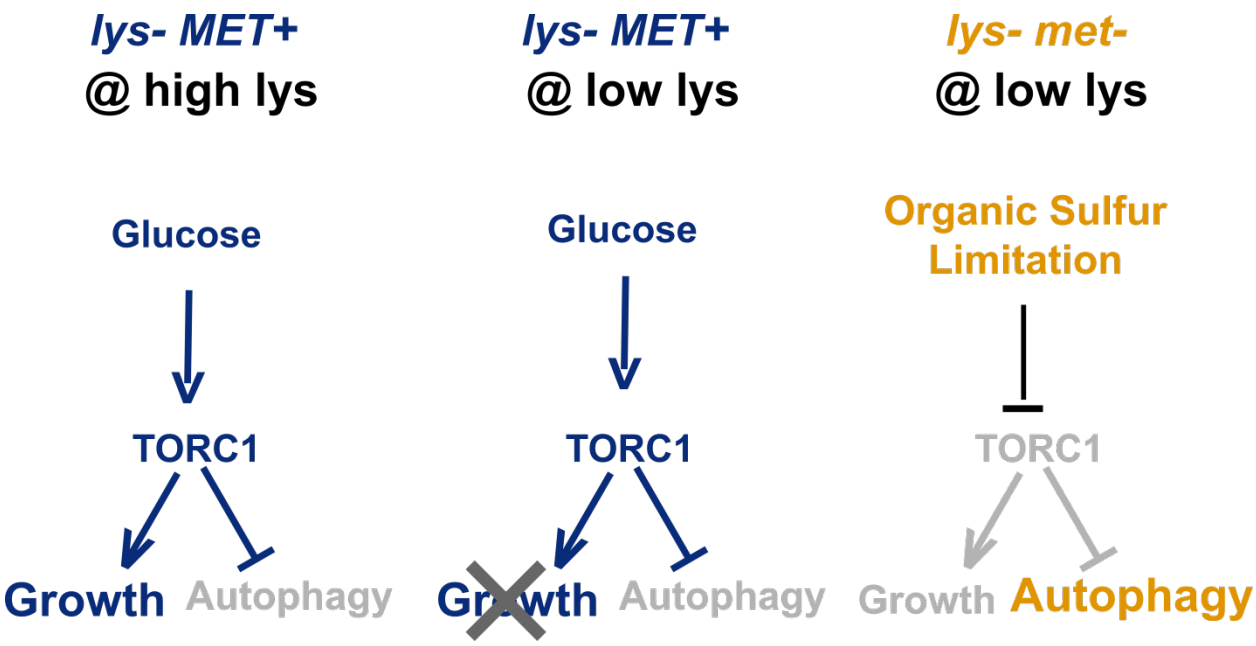

Figure 22 – Differing TORC1 regulation can explain the viability phenotypes for both organosulfur auxotrophs and prototrophs. Simplified TORC1 diagrams for organosulfur auxotrophs and prototrophs in the **[L-]** background.

Chapter 5. EVOLUTON OF A SIMPLE METABOLIC COMMUNITY IN RESPONSE TO MISREGULATED NUTRIENT SIGNALING IN YEAST

5.1 YEAST LYSINE AUXOTROPHS AS A MODEL FOR TORC1 MISREGULATION

The purpose of this thesis was to understand how a misregulated system of TORC1 signaling could adapt using experimental evolution. TORC1 is considered a 'master regulator' of nutrient signaling[16] and is responsible for regulating generating a 'proper' response to nutrient availability. Yeast has been used as a model system to study physiological responses to nutrient availability. Through earlier work, it was demonstrated that certain auxotrophs, include lysine, seemed to bypass TORC1 regulation when limited for the amino acid or nucleotide the strain required[14]. These strains wasted glucose, failed to properly regulate cell cycle arrest, and had greatly diminished viability[4]. Transcriptomic analysis revealed that lysine auxotrophs had a drastically different physiological response to lysine limitation compared to prototrophs being limited for a natural limitation like glucose.

In this chapter I will discuss my results in the process for understanding the evolution of a misregulated TORC1 system. In Chapter 6, I will argue that despite this system being fundamentally broken or 'artificial', ecological insights can still be gained through its study. For the duration of this chapter, I will focus on the evolutionary novelty of this broken system fixing itself in a non-intuitive manner.

5.2 Adaptation to Lysine Limitation follows a Predictable Trajectory … at First

When adapting to nutrient limitation, typical 'first steps' of evolution is to select for mutations that enhance the affinity for the limiting nutrient [24,83]. As expected, the **[L-]** lineage quickly became dominated by clones with higher amounts of the lysine permease Lyp1 on their cell membrane by either negating membrane protein turnover or increasing copy number of the *LYP1* locus through aneuploidy. Initially, this appeared to be a trivial result. However, during sequencing and phenotypic analysis, I found that roughly 10% of the clones I was screening had become auxotrophic for additional metabolites (glutamine and organosulfur) that had not been supplied exogenously. This was a remarkable finding: evolution of auxotrophy in the absence of required metabolites had never been reported for eukaryotic organisms.

5.3 There is a Quantitative Link Between Lysine Metabolism and Glutathione Release that Leads to Ecological Consequences

I reasoned that because no organosulfur compounds were supplied exogenously, the **[L-]** organosulfur prototrophs must be supplying it. This is known as niche construction[84]. I demonstrated that not only was glutathione being released from live cells, but ultimately there was a quantitative relationship: the more lysine limited populations were, the more glutathione was released. This allowed for the support of organosulfur auxotrophs.

What is fascinating is that the lysine auxotrophy adapting to lysine limitation constitutes a broken system because of the misregulation/bypassing of TORC1. However, the brokenness of the system is also what appears to be driving this ecological interaction. By imposing a greater degree of lysine limitation, which could be thought as stressing the broken system more, there is

more of an ecological opportunity for auxotroph evolution. Whether this is due to metabolite imbalance, redox stress, or something pleiotropic with TORC1 that I haven't considered, the pattern is fascinating: the more stress the system is under, the greater chance for fixing itself through ecological forces.

5.4 A Glutathione Niche Allows for Restoration of TOR Signaling and a Return to a More 'Natural' Metabolic State

When the organosulfur niche was present, it could be realized by strains acquiring simple loss of functions in sulfur fixation. Because organosulfur is sensed by TORC1, becoming an auxotroph is equivalent to inhibiting TORC1, provided that organosulfur concentration is low enough such that there is some probability that an organosulfur auxotroph is physiologically limited for organosulfur. This drastically lowers the death rate of auxotrophs because TORC1 is now off and will signal for the cell to enter a more dormant state. Because of this difference in death rate between auxotrophs and prototrophs, auxotrophs can increase in frequency to the extent that they do not completely abolish their niche. They can therefore exist with organosulfur prototrophs in a negative frequency-dependent manner, where the two players in the interaction differ in their metabolic strategies.

5.5 In Response to A Miswired Genetic Architecture, a Simple Community is Formed

TORC1 is one of the most important regulators of nutrient availability and physiology in all eukaryotes. Its misregulation has implicated in many human diseases such as cancer[17]. Many studies have characterized how TORC1 misregulation could alter human physiology at the cellular level[72]. However, to my knowledge, no study has looked at how TORC1 misregulation

could adapt over evolutionary time. This question is relevant not only for understanding biological fundamentals, but also tumor evolution in the clinic. I chose a lysine auxotroph of *S. cerevisiae* as a model for TORC1 misregulation and evolved it under lysine limitation. Along with the expected mutations that enhance lysine affinity, many clones had gained mutations making them auxotrophic for organosulfur compounds not supplied in the media. As a function of lysine limitation, organosulfur prototrophs released glutathione that supported organosulfur evolution. It is likely that other compounds in the organosulfur niche are glutathione-based. This supported organosulfur auxotroph evolution. These new auxotrophs rose to high frequencies because of a selective advantage, which was mediated though restoration of TORC1 signaling resulting in greater viability than their competitors (Fig. 23).

This result is profound because rather than simply selecting for suppressor mutations, as one would expect, lysine limitation of a lysine auxotroph is a self-correcting system. Lysine limitation of a lysine auxotroph drives the release of metabolites from live cells that allows for organosulfur auxotrophy to persist. Given that many tumors are heterogeneous and engage in metabolic trade[85], this could be a simple scenario for the maintenance of tumor diversity. Many cancers are known to be auxotrophic for various compounds, such as arginine[86] and methionine[87]. Why would tumors become auxotrophic for such essential metabolites when proliferative growth is very demanding for resources? On possibility is that as tumors expand, metabolic misregulation can lead to metabolite release into the environment. This leads to specialization which can be facilitated by metabolic trade. If tumors could stabilize TORC1 misregulation in a similar manner, it would allow for greater persistence in conditions where growth is not possible due to resource limitation.

I hypothesize that suppressing deleterious phenotypes through nascent social behavior may be an underappreciated aspect of tumor robustness. Indeed, the social aspects of tumors with hosts and symbiotic microbes has recently become a topic of interest[88]. A driving force behind this social aspect of tumor evolution could be related to the metabolic imbalance brought on by TORC1 bypassing and misregulation.

5.6 FIGURES

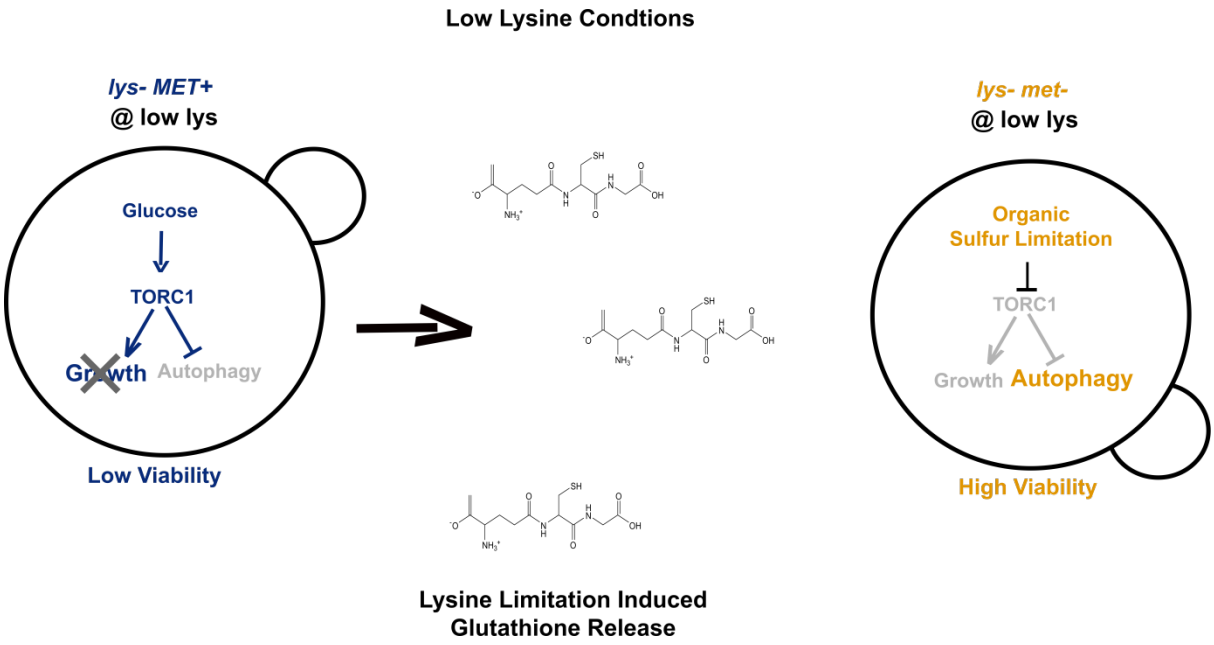

Figure 23 – Stress-induced metabolite release creates an ecological and evolutionary opportunity for restoring growth regulation. Lysine-limitation drives the release of glutathione from **[L-]** organosulfur prototrophs, which supports **[L-]** organosulfur auxotrophs. Organosulfur auxotrophy results in reacquisition of TORC1 signaling, which promotes autophagy and drives an adaptive advantage due to higher viability.

Chapter 6. A SIMPLE MODLE FOR THE EMERGENCE OF METABOLOIC DEPENDENCIES AND POTENTIAL EVOLUTIONARY CONSEQUENCES

6.1 WHY STUDY THE EVOLUTION OF METABOLIC DEPENDENCY?

Microbes are inherently social creatures, often living in complex, heterologous communities. Microbial communities have recently gained increased interest in the scientific community due to their ability to perform useful functions[89] and exhibit complex behaviors (robustness)[90]. Understanding the nature of these communities will yield key insights into not only evolutionary and ecological driving forces that shape natural environments, but also potential ways to engineer these communities for industrial and biotechnological purposes.

One important aspect of microbial communities is the effect one member has on another member's fitness or physiology. I define a *microbial interaction* as anything that affects the fitness or physiology of another microbe beyond competition for resources. One form of interaction is physical aggregation of microbes in a biofilm through the formation of an extracellular matrix [91]. Experimental evidence has shown that this can lead to a syncing of physiology between the inside and outside of the film [92]. Another example is the excretion complex public goods such as of invertase [93] or iron-scavenging siderophores[94]. In both cases, these goods can be excreted by a single species but benefits can be reaped by multiple species, even non-producers. Public good excretion can also be harmful. For example, many bacteria can participate in chemical warfare such as bacteriocin production, which kills competitor cells that don't contain the correct immunity protein [95]. Interactions can also be important drivers of sexual

reproduction, both through the production of mating pheromones [96] as well as direct exchange of genetic material[97].

An important form of interactions is the release and uptake of metabolites from one species to another, known as *cross-feeding*. These metabolites can be waste products [98] or costly (meaning the producer had to pay fitness cost to release the metabolite) [99]. Cross-feeding is readily observed in natural communities such as the soil [100], ocean [52], and human gut[101]. It has also been studied in laboratory evolution experiments using standard lab organisms, such as *E. coli* and *S. cerevisiae* [102] [103] [23]. Finally, there is a slew of theoretical work that attempts to explain these behaviors in the context of a cost/benefit analysis[99] [54] [104].

An extreme form of cross-feeding is metabolic dependency. Well-studied cases of metabolic dependency have recently appeared in the literature. For example, many strains of algae lack the required biosynthetic machinery to produce vitamin-B12, which is essential for, among other things, the synthesis of the amino acid methionine[105]. Phylogenetic analysis suggest that gene loss events drove the emergence of auxotrophy and that this occurred across many independent lineages[106]. It was recently demonstrated that algae can fulfill their vitamin-B12 requirement through symbiosis with marine bacteria[52]. Another well-studied example of wild-auxotrophy is the SAR11 clade of marine bacteria, one of the most dominant forms of bacteria on earth, often comprising up to 30% of the total biomass in certain areas [107]. SAR11 is characterized by a highly-reduced genome[108] and as a consequences lacks many biosynthetic functions. Studies have shown that SAR11 requires reduced sulfur[51], glycine [109], serine[109], and vitamin-B1[110]. Since much of the ocean is nutrient deficient [53], it is likely that SAR11 receives these required metabolites from other bacteria. Finally, metagenomics analysis of microbiomes harvested from different environments suggests that metabolic dependency may be more of a

rule than an exception: many species are predicted to lack the required genes for at least one amino acid[50,67,111,112]. The ubiquity with which metabolic dependencies are found in the wild suggest that these behaviors are important in natural microbial communities.

Altogether, there is ample evidence to suggest the understanding of microbial interactions can be gleaned by studying the emergence of metabolic dependency. First, we can ask how might auxotrophy arise in nature?

6.2 Theoretical Models for the Emergence of Metabolic Dependency

The ubiquity of metabolic dependency poses an interesting evolutionary question: why would species lose their autonomy so readily when metabolic dependency limits their ability to survive in isolation? How do these auxotrophs become so abundant? In order to address this question, I propose that there are two crucial factors that must be explained in the evolution of metabolic dependency: 1) a supply source of metabolites that support auxotroph and 2) an evolutionary driving force that allows auxotrophs to become abundant.

First I consider the source of metabolites. One possibility is, rather than engaging in metabolic trade, species just happen to inhabit environments that are nutrient rich, such as industrial bioreactors[113] or the cytoplasm of host cells (in the case of endosymbionts)[114,115]. However, many environments are likely nutrient-poor. Another possibility is that populations of competing microbes will have winners and losers and when a cell is unable to get the required nutrients, it will die, lyse, and release its internal metabolites [116,117,38]. Species could release metabolites as part of normal physiology, such as the excretion of waste products[102]. Finally, organisms could produce metabolites, such as amino acids, that are essential but are unable to retain these metabolites within their cellular membrane due to concentrations inside the cell

being higher than outside the cell. This would great a thermodynamic push for metabolite 'leakage', either directly through the cell membrane [118] or membrane channel proteins [119]. This last posited to be an important part of the recently coined 'Black Queen Hypothesis'[120] and has been stated in the literature as 'Black Queen interactions'[121].

Once the source of metabolites is present, a cell that loses its ability to synthesize that metabolite can be supported. Next, in order for the new auxotroph to become common, it must increase in frequency in the population. The relative strength of different evolutionary forces driving genotypic frequencies depend on the population size. In small populations, such as those of endosymbionts constrained to host cells, drift is likely the driving factor of auxotroph evolution. The nutrient rich environment of the host cytoplasm creates relaxed selection for metabolic independence while the vertical transmission introduces tight population bottlenecks. These conditions can result in the emergence of auxotroph simply due to chance [114]. For larger populations, drift becomes less important and other driving forces must be used to explain the rise of auxotroph frequency. One possibility is auxotroph could carry a selective advantage, either through energy savings[81], redistribution of metabolic fluxes[122], or some additional pleiotropic effect[123]. Another possibility is that auxotrophy is neutral or deleterious, but it happens to emerge in a background with a highly beneficial mutation, so auxotrophy can hitchhike to high frequencies. Finally, auxotrophy may be deleterious and is weeded out by selection, but could emerge by mutation at a fast enough rate that selection cannot effectively remove auxotrophy from the population. In this case, there would be an equilibrium frequency that auxotrophy is maintained. This is known as mutation-selection balance[124].

Understanding these two factors (the source of required metabolites and the mechanism by which auxotrophs rise to high frequencies) are critical to understanding auxotroph evolution.

However, many natural microbes are difficult to work with and lack molecular tools for mechanistic analysis. Instead, we can resort to experimental evolution of laboratory systems to gain insight.

6.3 Metabolic Dependency in Other Studies

The above scenarios outline the *theoretical* ways auxotrophy could emerge in microbial populations, but are there situations where one is more likely than another? Working with naturally occurring auxotrophs is a challenge, as many come from environments where defined laboratory media is not available. As a complementary approach, molecular genetics can be used to construct auxotrophic strains in model systems and characterized by physiological studies. For example, Dykuizen and D'Souza *et al.* demonstrated that synthetically constructed auxotrophs could be selectively advantageous in nutrient-rich environments[66,67]. Experimental studies have also demonstrated that auxotrophs can persist in environments lacking an exogenous supply of the required metabolites. D'Souza *et al.* showed auxotroph evolution of *E. coli* in in minimal media that transiently persisted[125] while Campbell *et al*. showed maintenance of auxotroph cross-feeding pairs driven by plasmid loss in *S. cerevisiae* [49]. While these studies are excellent examples of auxotroph evolution under laboratory conditions, the mechanistic aspects outlined above are were not elucidated.

6.4 This Study as a Model for Metabolic Dependency Evolution

The unique aspect of this work relative to previously published studies is that I have delineated mechanistic aspects for both the source of metabolites to support auxotroph evolution and the fitness advantage of auxotrophy to a cellular level. To my knowledge, this study is one of

the first examples of evolution of a non-engineered interaction being characterized to this degree. I believe this system provides generalizable conclusions about the nature of microbial interactions. Herein I will argue that this system, while 'artificial' in the sense that yeast lysine auxotrophs lack regulation required to robust survival compared to a prototroph, the patterns that have emerged from this study in understanding both the ecological niche generation and the adaptive benefit of gene loss are just as relevant as any study to date.

6.5 Metabolic Niches Can Be Generated in Response to Environmental Stimuli and Cellular Genotype

By definition, a microbial interaction facilitated by a metabolite or public good must be produced in one cell, released, and taken up by another. This leaves a simple but so far unanswered question in evolutionary theory: why would metabolite/goods be released in the first place? What drives the release of metabolites at the inception of an interaction?

A common problem in understanding the nature of microbial interactions is the cost of producing a good that will be distributed to the external environment[99]. If a good/metabolite is generated as a result of metabolism and the producer pays no cost (does not become less fit) to release it to the environment, then there is no conflict between what's best for the producer and what's best for the rest of the players involved in the interaction. This is known as syntrophy. Syntrophy has been shown to evolve in several experiments with *E. coli* evolving under glucose limitation, where a subpopulation gains mutations that allow for better scavenging of glucose but result in the release of acetate[102, 37, 126,127]. Yeast have also been shown to excrete waste products in the form of amino acids when provided with excess nitrogen[55]. Yeast have also been found to release CO_2 from respiration when provided with excess glucose[128]. In these cases, waste

products are released because the environmental niches that are filled result in overflow of certain by-products that must be released.

For goods/metabolites that are costly to produce, the reason for their release into the environment is less clear. Why would a cell make itself less fit in order to produce a good/metabolite it won't be able to fully use[129]? One possibility is a benefit is gained by releasing this compound and the benefit gained can override the cost of production. An example of this is the production of invertase production in yeast[93,130]. Invertase is used to degrade sucrose to glucose that can be taken up and must be released to the environment. Work by Gore *et al*. showed that if a small fraction of glucose (1% of the theoretical value) is received by a producer, the benefit received is enough to outweigh the cost[93]. In this case, there is a selective pressure for the release of invertase (cells cannot grow if no invertase is made). However, whether this was the case at the inception of invertase utilization is not clear.

Ultimately, whether a good or metabolite is produced as a waste product or one that carries an additional cost, the reason for its export must be investigated. In this study, the severe lysine limitation that **[L-]** drove the release of glutathione, as the same strain grown under conditions of lysine excess released glutathione at a two-fold lower rate (Fig. 13). It is likely that glutathione can be categorized as a waste product, as this interaction evolved in a well-mixed environment[131]. This relationship between the excretion of a likely waste product and nutrient limitation is in contrast to previous studies, where the excreted byproduct the result of excess carbon or nitrogen[55] . It's possible that given that the sulfate in the media used in this study (5 g/L) is in excess of what the cell needs and must be released. However, glutathione is also an important redox regulator[132,133]. Two additional amino acids, glutamate and glycine, must be synthesized and incorporated for every glutathione produced. Additionally, my data suggest that

most if not all of the organosulfur niche is glutathione based (See Section 3.5 and Fig. 17). Why would glutathione and processes that create it and similar compounds be so targeted? Does nutrient limitation make some processes more 'wasteful' and is energy diverted inefficiently because a cell isn't adapted to this limitation? Is the redox state of a cell something that can have ecological impacts?

I believe that this study is an example of metabolite release being more complicated than waste release or cost/benefit optimization. The quantitative nature being lysine limitation and glutathione release is not well understood. Neither is the reason the cell has on releasing glutathione-related compounds. This system provides an excellent opportunity to look at 1) how environmental stimuli can generate ecological forces and 2) how specific biological modules have ecological consequences. With the advancement of 'omics' technologies, I believe that this is a next important step in the field of microbial ecology.

6.6 Frequency-Dependent Fitness Based Interactions can Establish Easily

In order for metabolic dependencies based on microbial trade to persist, there must be a mechanism by which the auxotroph does not outcompete the prototroph. This can be done through negative frequency-dependent fitness advantage[126, 80], which have been shown to evolve pervasively in evolutionary studies[55,39]. The molecular mechanisms by which these interactions form are less understood. In this case, environmental stress creates a niche that can be exploited through a one loss-of-function mutation in the right place. Previous studies have argued that metabolite release and utilization must be "tuned" and typically require a long amount of

evolutionary time [56, 134]. The ease at which this interaction formed argues that this may not be the case. Under the right scenarios, interactions can arise rapidly.

6.7 The Adaptive Benefit of Auxotrophy May Not Simply Be Due to Energy Savings and Is Likely Due to Pleiotropy

Many mutations that are sampled by evolving cells are likely loss of function, yet in experimental evolution studies loss-of-function mutations seem to be adaptive[64,135]. Indeed, a recent systematic analysis of gene-knockouts show adaptive loss of function mutations to be highly enriched in haploid yeast[69]. Additionally, this same study demonstrated that adaptive loss-of-function mutations between one condition (such as yeast grown in sulfate limitation) could greatly differ relative to another condition (such as yeast grown in glucose limitation)[69].This is known as antagonistic pleiotropy[136]. Other studies with yeast have shown that loss of function mutations that target information processing can be adaptive in a relatively constant envinronemnt[135].

The fact that the fitness effect of a mutation is 1) dependent on the external environment and 2) has downstream effects on pathway function (in terms of information processing), would argue against the explanation that metabolic dependency can be explained simply by energy savings [81,67]. This study demonstrated that the adaptive benefit of auxotrophy could be explained by pleiotropic effects on the regulation of metabolism and nutrient sensing. A common theme for many microbes in the wild is dormancy phenotype[137 ,138]. Given that metabolism has been predict to be very important in the epistatic effects of mutations[123], it's reasonable to hypothesize that metabolic dependency in natural systems could be due to effects on regulation of metabolism and stress response which could contribute to a dormancy phenotype. Indeed, auxotrophy has been

shown to be important in the maintenance of persister cells (cells that are highly robust to environment stresses such as antibiotics)[139].

This study provides clear evidence that auxotrophy can have non-intuitive effects on metabolism. While this was demonstrated in a yeast system, I argue that molecular parsing of the adaptive benefit of any system based on gene-loss, especially those related to cross-feeding, is required in order to fully understand how the system works.

Chapter 7. CONCLUSIONS

The purpose of my thesis was to understand how a misregulated system of nutrient sensing and regulation could adapt. Lysine auxotrophs of *S. cerevisiae* seem to bypass the important nutrient regulator TORC1 (Fig. 1), which results in a wasteful metabolic phenotype[14] and fast death (Fig. 2). To study this, I evolved a lysine auxotroph of *S. cerevisiae* under various conditions of lysine limitation (Fig. 3). Evolution took place in either a monoculture of a lysine limited chemostat or in a coculture with a lysine-releasing strain where growth was coupled to growth of the [L-] strain. In this system, the low lysine levels would result in the TORC1 being on even when lysine was not abundant, resulting in a population with high turnover.

In Chapter 2, I demonstrated through sequencing analysis that, as expected, a common target of adaption was to increase the affinity for lysine through loss of function mutations in the ubiquitination and turnover of the lysine permease Lyp1[140] or through chromosome duplication resulting in an increased copy number of the *LYP1* locus. Additionally, I identified a unique phenomenon: in roughly 10% of clones sequenced, many seemed to carry loss of function mutations in genes essential for the biosynthesis of organosulfur and glutamine (Table 2). Phenotypic analysis revealed that these clones were indeed auxotrophic for these compounds,

despite that they were not exogenously supplied in the evolution experiment. I designed the rest of my thesis to characterize this phenomenon, with a focus on organosulfur auxotrophy as it was the most prevalent in all auxotrophs identified.

In Chapter 3, I sought to identify the source of nascent metabolites that could support organosulfur auxotroph evolution. Since auxotrophy evolved in the monoculture conditions, I hypothesized that the **[L-]** strain was capable of releasing metabolites that could support auxotroph evolution. Growing the ancestor under lysine limitation and harvested supernatants for spent media analysis demonstrated this was the case (Fig. 6) Mass spectrometry (Fig.8) and bioassays (Fig.9) revealed glutathione in the reduced form as an important molecule in the organosulfur niche. Additionally, preliminary bioassay data suggested that glutathione was likely a predominate compound (Fig.9). This same assay revealed that there appeared to be a difference in pool size between supernatants harvested from lysine-limited populations (in the form of an 8 Hr doubling) and lysine rich populations (in the form of populations grown in a turbidostat grown at maximal growth rate). I hypothesized that there was a quantitative relationship between the degree at which populations are stressed for lysine and the amount of glutathione they released. To test this, I developed a quantitative HPLC assay (Figs. 10-12), which revealed that there was a 2-fold difference in release rate of glutathione between lysine-limited and lysine-rich populations (Fig. 13), indicating that the lysine-limitation *itself* was driving the release of glutathione. To determine if cell lysis could explain the release of metabolites, I developed a quantitative metabolite extraction protocol to assess how much glutathione could be attributed to each dead cell in my chemostats. This analysis revealed that cell lysis alone cannot explain all of the glutathione released, indicating that there must be a live-cell release component (Fig. 14). Finally, I developed a bioassay to assess the total pool of organosulfur, which indicated that

reduced glutathione could explain only 1/3 of the total niche. However, a quantitative assay using a strain incapable of using glutathione (*dug1*) revealed that this pool is likely still glutathione based, but in a different form (such as a large polymer or complexed with a peptide). This data altogether a quantitative relationship between metabolite release and environmental stress can rapidly generate ecological opportunities for adaption.

After explaining the nascent source of metabolites, I sought to address how auxotrophs rose to become some common in these evolved populations. I hypothesized that given how many times auxotrophy emerged that there may be an adaptive benefit. To test this, I performed isogenic competitions of an organosulfur auxotrophs and organosulfur prototroph under lysine limitation and demonstrated that organosulfur auxotrophy was adaptive in a negative frequency-dependent manner (Fig. 18). I then developed an assay to test if the adaptive benefit could be explained by the popular argument of energy savings or could be explained via a change in viability. This adaptive benefit could not be explained by the prevailing hypothesis of energy savings (Fig. 20). Organosulfur auxotrophy confers enhanced viability under limitation for lysine and glutathione (Fig. 21). This appears to be mediated through the restoration of TORC1 signaling, as treatment with rapamycin rescues the death of organosulfur auxotrophs in the presence of glutathione (in the absence of lysine) and abolishing autophagy, a known downstream process of TORC1, diminishes the survival phenotype.

Altogether, in response to a misregulated TORC1, the **[L-]** system was able to exploit the fact that lysine limitation drove the release of metabolites. This niche allowed for the adaptive nature of organosulfur auxotrophy to rise and persist. Basically, a system that is broken can correct itself through the evolution of novel metabolic interactions.

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VITA

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