Carbon and Nitrogen Content and Isotopic Composition of *Heliomicrobium Modesticaldum* Under Different Growth Conditions

by

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A Thesis Presented in Partial Fulfillment
of the Requirements for the Degree
Master of Science

Approved November 2021 by the
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ARIZONA STATE UNIVERSITY

December 2021

ABSTRACT

All known life requires three main metabolic components to grow: an energy source, an electron source, and a carbon source. For energy, an organism can use light or chemical reactions. For electrons, an organism can use metals or organic molecules. For carbon, an organism can use organic or inorganic carbon. Life has adapted to use any mixture of the endpoints for each of the three metabolic components. Understanding how these components are incorporated in a living bacterium on Earth in modern times is relatively straight forward. This becomes much more complicated when trying to determine what metabolisms may have been used in ancient times on Earth or potential novel metabolisms that exist on other planets. One way to examine these possibilities is by creating genetically modified mutant bacteria that have novel metabolisms or proposed ancient metabolisms to study.

This thesis is the beginning of a broader study to understand novel metabolisms using *Heliobacteria modesticaldum*. *H. modesticaldum* was grown under different environmental conditions to isolate the impacts of energy, electron, and carbon sources on carbon and nitrogen isotope fractionation. Additionally, the wild type and a novel mutant *H. modesticaldum* were compared to measure the effects of specific enzymes on carbon and nitrogen isotope fractionation. By forcing the bacterium to adapt to different conditions, variation in carbon and nitrogen content and isotopic signature are detected. Specifically, by forcing the bacterium to fix nitrogen as opposed to nitrogen incorporation, the isotopic signature of the bacterium had a noticeable change. The mutant *H. modesticaldum* also had a different isotopic signature than the wild type.

Without the enzyme citrate synthase, *H. modesticaldum* had to adapt its carbon metabolic cycle, creating a measurable carbon isotope fractionation. The results described here offer new insight into the effects of metabolism on carbon and nitrogen fractionation of ancient or novel organisms.

ACKNOWLEDGEMENTS

I would like to give thanks to Dr. Hilairy Hartnett who has helped me through this arduous process and for looking out for what was best for me during my time at ASU, as well as in the future. I would also like to thank the CaNDy lab members, specifically Josh Nye and Donald Glaser who not only helped me through understanding individual problems, but also helped me get through graduate school as a whole.

I would also like to thank the Redding lab, specifically Patricia Baker and Alexandria Layton who grew the cells for my project and were able to create the novel mutant bacteria.

A special thanks to Natasha Zolotova and the METAL lab at ASU for helping me analyze my samples and adapt to my changing timeline. I would also like to send a special thanks to Subhadeep Dutta and the Rege lab at ASU for allowing me to preform the lyophilization of my samples.

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CHAPTER 1

INTRODUCTION

Astrobiology and Biosignatures

In future expeditions and observations, a key element to determining the presence of life on other systems is looking for biosignatures. This can range from the presence of oxygen to unique isotopic signatures of bio-essential elements. On Earth, our atmosphere is 21% oxygen. This is due to the "Great Oxidation Event" that occurred 2.4 billion years ago (Catling, 2014). It is theorized this event was driven by life. Without life on Earth, oxygen may still be a trace element in our atmosphere. By analyzing the UV spectra from a distant star's light as it passes through a planet's atmosphere, the composition of an exoplanet's atmosphere can be determined (Seager, 2014). If oxygen is present in large quantities, it may be an indication of life on that planet. Another method for determining the possible presence of life is looking at the isotopic signature of bio-essential elements. On Earth, life favors ^{12}C over ^{13}C, resulting in isotopically depleted organic material compared to the far abundant inorganic carbon that is present in the atmosphere and in rocks (Vieth and Wilkes, 2010). If future expeditions to planets or moons in our solar system determine the presence of isotopically light carbon, it may indicate the presence of life.

On Earth, oxygen and carbon isotopes provide evidence for life by their difference between abiotic and biotic systems. However, we are limited to measuring

biosignatures on Earth by the life that is present here. Life may potentially have evolved differently on Mars or exoplanets and could have different signatures. To study future samples and account for the possibility of different signatures, novel biological systems must be created. One way to create a novel biological system is to create a mutant bacterium. Creating mutant bacteria is done for many reasons already, but it is not done with the desire to create novel biological systems within the bacteria. It is necessary to manipulate known organisms and their metabolisms to determine if different organisms and metabolisms can sustain life in a way that is unique to life beyond Earth. There are three main types of metabolism types to an organism: Energy source, reductant source, and carbon source (Fig. 1). Each of these three sources has two major categories. The energy source for an organism can come from light (photo) or chemical reactions (chemo). The reductant source can come from metals (litho) or organic molecules (organo). The carbon source can come from inorganic carbon (auto) or organic carbon (hetero). An organism will fall into one of the two categories for each type of metabolism. The classification of this organism is then listed as the concatenation of these naming conventions. For example, humans are chemoorganoheterotrophs. By taking an organism and changing one of the metabolisms from one category to the other (ex. photoorganoheterotroph to chemoorganoheterotroph), would create a novel mutant. This novel mutant may be distinguishable by isotopic signature.

For each of the metabolisms, minor changes within a category can also be made. For example, an organism may strictly be a chemotroph, but could utilize light and chemical reactions as a source of energy if given the genes to create a photocomplex.

This may not necessarily make the organism an autotroph, as it is possible for an organism to utilize light but not able to do photosynthesis. Another change could be to alter the steps in a known metabolism to determine the effect on the organism. If the gene for citrate synthase was excluded from a mutant organism, the rTCA cycle would become a linear metabolic pathway and possibly result in a different biosignature for the mutant compared to the wild type.

At first, small changes to an organism may not yield vastly different biosignatures, but continual changes may lead to a new biosignature that could hypothetically have naturally evolved on another planet. My project is a first step in investigating this process.

Why Heliomicrobium

Heliomicrobium modesticaldum is a well-studied photoorganoheterotroph bacteria, so its capabilities and required growth conditions are known (Baker et al., 2019b; Kimble et al., 1995). Although *H. modesticaldum* has been studied, its isotopic composition has not been measured. *H. modesticaldum* is a unique microbe because it is the only known anaerobic anoxygenic phototroph that is not an autotroph (Asao and Madigan, 2010). In general, wild type *H. modesticaldum* must be grown under anoxic conditions. Heliobacteria are currently the only known organisms to use bacteriochlorophyll g which, if exposed to oxygen, will react to create bacteriochlorophyll a (Oh-oka, 2007). *H. modesticaldum* cannot use bacteriochlorophyll a, and would die if exposed to oxygen. Heliobacteria are also unique as the only phototrophic bacteria within the Firmicutes phylum (Sattley et al., 2014). Although *H.*

modesticaldum is a phototroph, it does not fix CO_2 using light, and its photochemical reactions do not produce molecular oxygen as a byproduct. Although *H. modesticaldum* uses light, it is not an autotroph and must use organic carbon to grow, making it a heterotroph (Asao and Madigan, 2010; Blankenship, 2014; Sattley et al., 2014). *H. modesticaldum* has the mechanisms for both phototrophic growth, as well as chemotroph growth using pyruvate fermentation as the energy source (Baker et al., 2019b). The primary carbon metabolism used by *H. modesticaldum* is the TCA cycle (Fig. 2). *H. modesticaldum* can utilize both the forward and reverse directions of the cycle (Hohmann-Marriott, 2014; Tang et al., 2010). For nitrogen, *H. modesticaldum* can fix N_2 but will preferentially use a reduced nitrogen source such as NH_4Cl or glutamine when one is available. When provided an alternative nitrogen source in the media, the nitrogen fixation pathway is turned off (Baker et al., 2019b; Blankenship et al., 1995).

The genome of *H. modesticaldum* is known, and the Redding group has developed the tools to modify its genome using the organism's own crisper system (Baker et al., 2019b, 2019a; Sattley et al., 2008). The Redding group has successfully grown an *H. modesticaldum* mutant that lacks a citrate synthase (CS) enzyme. This forces the mutant to use a linear version of the rTCA cycle for carbon metabolism. I have analyzed the content and isotopic composition of the wild type and mutant *H. modesticaldum* to examine the difference between the two and the impact of the CS enzyme. This mutant, as well as any future mutants that can be grown could reflect the early stages of *H. modesticaldum* evolution and might be proxies for early earth metabolism or for potential metabolism on other planets.

Isotopic background

Measuring the wild type content and composition provides a baseline measurement for *H. modesticaldum* to which measurements of mutant *H. modesticaldum* can be compared to. *H. modesticaldum* is a heterotroph, so I expect the bacteria's carbon isotopic signature to resemble the isotopic signature of the carbon they are consuming, as seen in most heterotrophs (Boschker and Middelburg, 2002). Heterotrophic bacteria that consume C3-plants, C-4 plants and phytoplankton all had the same $\delta^{13}C$ values as the carbon they were consuming. The $\delta^{13}C$ values for all primary producers was different as they have different primary production carbon metabolisms (Boschker and Middelburg, 2002). There was a change in the $\delta^{13}C$ value (relative to the Pee Dee Belemnite standard, VPDB) between the autotrophic primary producers and the pool of inorganic carbon utilized (Posth et al., 2017). Once an autotrophic mutant of *H. modesticaldum* can be grown, it may have a varied isotopic signature depending on the type of autotrophic metabolism being used.

In general, the inorganic pools of carbon have an isotopic value between +2 ‰ and -8 ‰; marine carbonates are about 0 ‰ and atmospheric CO_2 is slightly depleted at -8 ‰ (Boschker and Middelburg, 2002). When autotrophic organisms use this carbon, it is fractionated due to biology's preference for the lighter isotopes (Fogel and Cifuentes, 1993; O'Leary, 1981; Spona-Friedl et al., 2020). This kinetic fractionation causes most organic carbon to be isotopically depleted relative to inorganic carbon.

Nitrogen isotopes also provide useful information about metabolism. There are five main biological processes involving nitrogen: nitrification, denitrification, nitrogen

fixation, ammonium assimilation, and nitrate assimilation. Each of these nitrogen processes impose a fractionation, resulting in a known range of isotopic values (Fogel and Cifuentes, 1993; Sigman and Casciotti, 2001). The two main reactions that *H. modesticaldum* uses are nitrogen fixation and ammonium assimilation. Bacteria that assimilate ammonium will generally exhibit a $\delta^{15}N$ value between -6.5 ‰ and -20‰ (Sigman and Casciotti, 2001). A nitrogen fixer will have an isotopic value of about 0‰ as this process does not fractionate nitrogen (Estep and Macko, 1984; Fogel and Cifuentes, 1993).

In this thesis I present the methods for growth and analysis of *H. modesticaldum* cells, as well as the data analysis conducted. I present results of the carbon and nitrogen content, the isotopic composition for each of the different growth conditions, and compare different growth conditions including: 1) the effect of being grown in the dark (to force chemotrophic growth), 2) growth using different carbon sources to change the amount of CO_2 that cells need to incorporate during growth, 3) the effect of nitrogen fixation on the nitrogen isotope composition, 4) and the content and isotopic compositional difference between the wild type and a mutant strain of *H. modesticaldum*. There are small differences seen between the wild type and mutant cells, but the largest content and composition changes were a result of forcing nitrogen fixation.

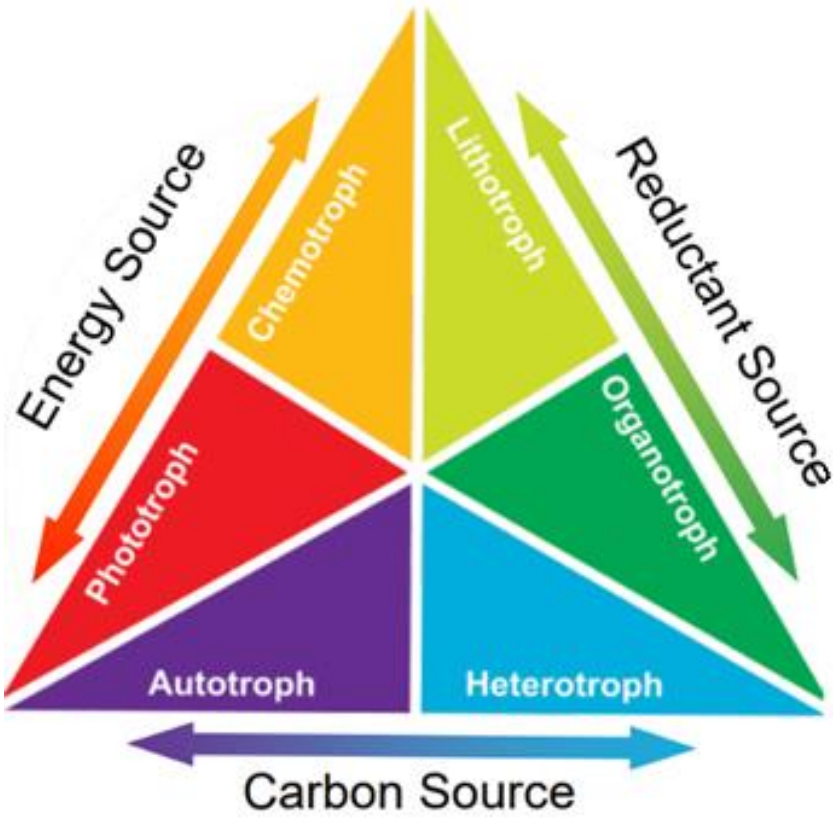

Figure 1.1. A simplified diagram illustrating metabolism types. Names for each side of the triangle can be combined to create one energy-reductant-carbon-'troph' name. An example is *Heliomicrobium modesticaldum* growing in the light is a photoorganoheterotroph. Figure from Garcia-Pichel 2005.

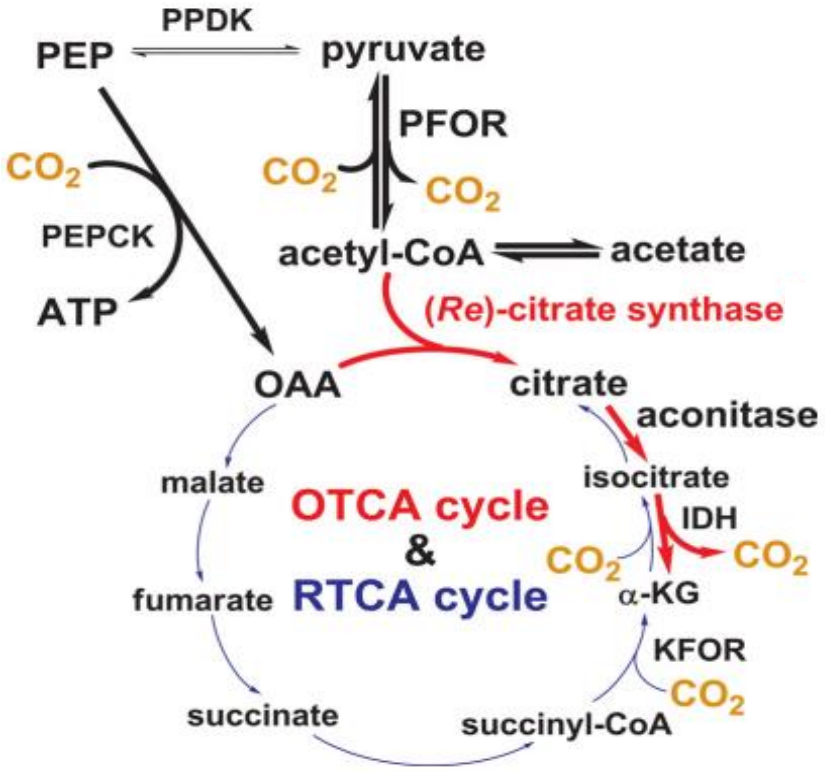

Figure 1.2. A diagram showing the oTCA cycle and rTCA cycle that is present in *H. modesticaldum*. Figure modified from Tang et al., 2010.

CHAPTER 2

METHODS

2.1 Culture Growth conditions.

H. modesticaldum was grown in 45 mL bottles in the Redding lab. The headspace was a mixture of 20% carbon dioxide, 75% nitrogen, and ~5% hydrogen by mass. Hydrogen is included to react with trace oxygen to ensure an anoxic environment and therefore H_2 fluctuated between 2 and 4%. All cells were grown at pH 7.4 and 22°C. Two different media were used as the base recipe for all 8 different growth conditions. These two base recipes are denoted by which growth conditions used yeast extract. Cells generally grow better when there is yeast extract in the media. The growth media that do not contain yeast have different salt concentrations compared to media with yeast. The base media is further adjusted to have different carbon sources (pyruvate or acetate), and different nitrogen sources (ammonium, glutamine, or N_2) The 8 different growth media and 11 different cell cultures, as well as the naming conventions are shown in table 1.1. Relationships between different cell cultures can be seen in figure 2.1.

The base recipe with yeast (PyA/Y) was grown under white light and included pyruvate (2.2 g/L), ammonium chloride (1.0 g/L) and yeast (4 g/L). Other minor ingredients included: K_2HPO_4 (1 g/L), $MgSO_4 \cdot 7\ H_2O$ (0.2 g/L), $NaS_2O_3 \cdot 5\ H_2O$ (0.2 g/L), $CaCl_2 \cdot 2\ H_2O$ (20 mg/L), Vitamin B12 (25 µg/L), and biotin (18.75 µg/L). Two additional growth conditions used this as the base recipe. A media that used pyruvate plus

ammonium plus yeast in the dark (PyA/YD) allowed us to determine the effect of light as an energy source. This growth condition can be directly compared with PyA/Y. Media that contained acetate plus ammonium plus yeast (AcA/Y) used acetate (2.2 g/L) as the carbon source instead of pyruvate. By changing the carbon source from pyruvate to acetate, I can determine if carbon source has an effect on the carbon or nitrogen content and composition. This growth condition can be directly compared with PyA/Y.

The base recipe without yeast (PyA) was grown under light and included sodium pyruvate (Py; 2.2 g/L) and ammonium chloride (A; 1.0 g/L). Other minor ingredients included: EDTA (3.5 mg/L), $MgSO_4 \cdot 7H_2O$ (200 mg/L), $CaCl_2 \cdot 2\ H_2O$ (75 mg/L), K_2HPO_4 (0.9 g/L), KH_2PO_4 (0.6 g/L), biotin (7.4 µg/L), vitamin B12 (11.2 µg/L), 4-amniobenzoic acid (30.6 µg/L), $FeSO_4 \cdot 7\ H_2O$ (5.6 mg/L), Na_2EDTA (10 mg/L), $MnCl_2 \cdot 4H_2O$ (0.8 mg/L), H_3BO_3 (0.4 mg/L), $NaMoO_4 \cdot 2\ H_2O$ (0.4 mg/L), $VOSO_4 \cdot 2\ H_2O$ (20 µg/L), $ZnCl_2$ (0.2 mg/L), $NiCl_2 \cdot 6\ H_2O$ (0.2 mg/L), $CoCl_2 \cdot 6\ H_2O$ (80 µg/L), $CuCl_2 \cdot 2\ H_2O$ (20 µg/L), $Na_2WO_4 \cdot 2H_2O$ (20 µg/L), and Na_2SeO_3 (2.2 µg/L). Six additional growth conditions used this as the base recipe. The nitrogen source was changed for two media. The second nitrogen source used in the media was glutamine. Pyruvate plus glutamine (PyG) has glutamine (10 mg/L) as the nitrogen source instead of ammonium. Changing the nitrogen source was done to eliminate the problems with volatilization of ammonium during freeze-drying. The PyG media was used to grow both the wild type (PyG/WT) and mutant (PyG/CSKO) cultures. For the wild type cells, this growth condition can be compared to PyG and AcA growth conditions. The third nitrogen condition used N_2 to force the cells to fix nitrogen. In the pyruvate plus N_2 (PyN) media, ammonium was

removed from the recipe. This forced the cells to use N_2 as the nitrogen source instead of ammonium. This growth condition can be compared to PyA and PyG growth conditions.

Three more media conditions were created by changing the carbon source for each of the three media above. By changing the carbon source from pyruvate to acetate, I can determine if carbon source has an effect on the carbon or nitrogen isotopic values. The PyA media was adjusted to create an acetate plus ammonium (AcA) media. The AcA media has acetate (2.2 g/L) as the carbon source instead of pyruvate. This growth condition can be compared to PyA. The PyG media was adjusted to create an acetate plus glutamine (AcG) media. The AcG media has acetate (2.2 g/L) as the carbon source instead of pyruvate and glutamine (10 mg/L) as the nitrogen source instead of ammonium. Changing the nitrogen source was done to eliminate the problems with volatilization of ammonium during freeze-drying. The AcG media was used to grow both the wild type (PyG/WT) and mutant (PyG/CSKO) cultures. For wild type cells, this growth condition can be compared to PyG and AcA growth conditions. Similarly, PyN was adjusted to create an acetate plus N_2 (AyN) media. The AyN media has acetate (2.2 g/L) as the carbon source instead of pyruvate and removed ammonium was removed from the recipe. This forced the cells to use N_2 as the nitrogen source instead of ammonium. This growth condition can be compared to AcA, AcG, and PyN growth conditions.

2.2 Culture Harvesting

Cell cultures were poured into pre-weighed 50 mL centrifuge tubes and spun at 4863 g for 20 minutes. The supernatant from the sample was transferred to another pre-

weighed 50 mL tube and frozen at -20°C. The tube containing the cell pellet was washed by resuspending the cells in 10 mL of 18.2 MΩ·cm water. The mixture was then centrifuged again at 4863 g for 20 minutes. The supernatant from washes was discarded. Cell pellets were washed 6 times each to ensure that no residual media was left on the cell surfaces. Initial evaluation of the carbon content of the washes indicated that a minimum of 5-6 washes was necessary to clean the cells (Fig 2.2). The cells were then resuspended in 10 mL of 18.2 MΩ·cm water and frozen at -20°C in a 15 mL tube. Ten mL of media that was never inoculated with cells was also added to a 15 mL centrifuge tube and frozen at -20°C. The centrifuge tubes containing cells, or media were individually weighed, removing any condensation just prior to the measurement. To remove residual water, all three sample types were stored for 3 hours at -80°C; the vials were then uncapped and covered with three layers of Kimwipe secured with a rubber band and placed in 600 mL beakers attached to a FreeZone 4.5 Liter -84C Benchtop Freeze Dryer. Samples were lyophilized for 48 hours to ensure all water was removed. Dried samples were capped and returned to storage at -20°C. Individual components of the media were placed in 2 mL Eppendorf tubes to be measured separately. Any components that were dissolved and lyophilized to imitate the media prep are indicated with a prime. For example, glutamine' was dissolved and lyophilized prior to measurement, whereas glutamine was measured straight from the bottle.

2.3 Sample Analysis

I estimated the carbon and nitrogen content of my cells based on published estimates for the elemental composition of bacteria (Lawford and Rousseau, 1996). I

estimated the carbon and nitrogen content for each media samples using their corresponding recipes. Using these estimates, I created 3-5 replicate samples by measuring out the estimated amount of material needed on a microbalance to ensure every sample would fall within the calibration ranges for carbon and nitrogen content. The replicate samples were placed in individual tin capsules that were then folded twice over so no material would escape.

A “blank” tin capsule with no material is also included in each sample tray, as well as an empty slot in the sample tray. This ‘capsule blank’ is used to account for trace carbon or nitrogen contamination in the tin capsules or reference gasses. Five standards were used: Acetanilide, tomato leaves, glycine-high, glycine-mid, and glycine-low. Acetanilide was used as an instrument check to verify the retention time and shape of the nitrogen and carbon peaks are where they are expected. This was done to ensure the instrument was running properly so that it could be stopped before any samples were lost. I used a tomato leaf standard at seven designated weights: 0.2 mg, 0.5 mg, 1 mg, 1.5 mg, 3.0 mg, 4.0 mg, and 5.0 mg. Using these known weights, I was able to create weight vs. area calibration curves for carbon content and nitrogen content. The tomato leaf standards were also used as an in-house isotopic standard to create linearity curves to account for material amount impacting isotopic value (see section 2.4). Finally, glycine at three known isotopic values (glycine-high, 8.94 ‰ $\delta^{13}C$, 40.64 ‰ $\delta^{15}N$; glycine-mid, -8.36 ‰ $\delta^{13}C$, 27.9 ‰ $\delta^{15}N$; glycine-low, -39.64 ‰ $\delta^{15}N$, 1.35 ‰ $\delta^{13}C$) were used as an in-house isotopic standard. The carbon and nitrogen isotopic values for the glycine standards are also known relative to the global standards. My measurements were adjusted to the global

Vienna Pee Dee Belemnite (VPDB) standard for carbon and to the global atmospheric air standard for nitrogen using the glycine standards as a reference.

After the samples and standards were weighed, put into the capsules, and placed in the sample tray I transported them to the instrument the same day for analysis. All samples were analyzed on a Costech Elemental Analyzer (EA) coupled to a Thermo Delta Plus Advantage Isotope Ratio Mass Spectrometer (IRMS) in the METAL laboratory at Arizona State University. This coupled instrument measures both carbon and nitrogen content and carbon and nitrogen isotopic compositions and can run 48 individual samples or standards per sample run. The samples in the autosampler were then purged with helium for 5 minutes. The mass spectrometer gas inlet valves were checked to ensure the valves would open and close properly. Once all the 'pre-run' instrument checks were complete, I started the instrument. Analysis takes about 10 minutes per sample and is run overnight. The instrument is kept continuously running unless no samples are going to be prepared for an extended duration of time.

Samples and standards were treated identically within the instrument. To obtain measurements, a sample was dropped from the autosampler into a combustion tube sitting in a furnace at 1020°C. The tin oxidizes to gaseous tin oxide. Combustion in the presence of oxygen converts solid phase carbon into CO_2 molecules and solid phase nitrogen into N_2 molecules. The CO_2 and N_2 are then separated by gas chromatography from each other, as well as from any additional gaseous compounds created during the combustion process. A TCD detector measures the amount of gas molecules present over time. Reference gasses are used to determine the retention times for CO_2 and N_2. The

area of the peak in the TCD detector output at the pre-determined retention times are used for the area counts of a sample. The area counts are converted to C and N mass using the tomato leaf calibration curve. The C and N mass content is expressed as C mass per dry mass of the sample (mg/mg dry weight, or weight percent; see section 2.4).

A small portion of the sample gas that has passed through the gas chromatograph is also sent through an inlet to the mass spectrometer. The gas is ionized by thermal ionization using a platinum filament. A magnet is set to a specific field strength which separates the ionized gas according to its mass to charge ratio (m/z). Molecules that have the same charge, but different masses will be bent around the magnet at different radii due to the difference in kinetic energies. When the ionized gas hits the Faraday cup detectors, a change in voltage is measured. The number of times the Faraday cup is struck is counted. Three Faraday cups are used at m/z values of 44, 45 and 46 for Carbon and 28, 29 and 30 for Nitrogen. Carbon dioxide with a ^{12}C will have a m/z of 44 while carbon dioxide with a ^{13}C will have a m/z of 45. The isotopic ratio of a sample is calculated using the counts from the two different Faraday cups and dividing by the same ratio determined for a standard; the data are expressed in permil (‰) notation. The delta notation equation for carbon is presented as an example (Eq 1):

$$\delta^{13}C\ (‰) \ = \ 1000\ \text{x} \left\{ \frac{\left(\frac{^{13}C}{^{12}C}\right)_{\text{sample}}}{\left(\frac{^{13}C}{^{12}C}\right)_{\text{std}}} - 1 \right\} . \qquad \text{(Eq. 1)}$$

2.4 Data Analysis

The first analysis preformed is calculating the carbon and nitrogen content for each sample. Area from the TCD detector is used to determine carbon and nitrogen content. First, I corrected the area counts for standards and samples by subtracting the area of the empty tin capsule. A seven-point carbon calibration curve from 100 µg carbon to 2000 µg carbon is constructed using the known concentration of carbon and the area counts of the tomato leaf standards. The unknown carbon concentration of the media and cells was then calculated using their known amount of material and measured areas. A seven-point nitrogen calibration curve from 10 µg nitrogen to 150 µg nitrogen was constructed using the known concentration of nitrogen and the area counts of the tomato leaf standards. The unknown nitrogen concentration of the media and cells was calculated using their known amount of material and measured areas. I calculated carbon and nitrogen content by dividing the measured amount of carbon or nitrogen by the total amount of material in the dried sample.

The second analysis I performed is calculating the carbona and nitrogen isotopic values for each sample. The initial isotopic values calculated from equation 1 for the standards and samples give the isotopic composition as $\delta^{13}C$ and $\delta^{15}N$. These values are not comparable to the global standards without accounting for dependencies on mass and normalizing the values for the glycine standards to the global standards. The isotopic composition as determined in continuous flow isotope ratio mass spectrometry is somewhat dependent on the amount of the element present in the sample. To account for and influence of mass on the isotopic value, linearity curves were constructed. The

linearity curves were constructed by comparing peak area count for nitrogen or carbon with the measured isotopic signature for each of the tomato leaf standards. A logarithmic equation is fit to the data; this ensures that measured isotopic values are comparable due to the elimination of error resulting from variation in the amount of material measured. The last step I preformed to analyze the data was to construct a normalization graph. The three glycine lab standards used as an in-house reference have been measured in conjunction with global standards in the past. Using the glycine-high and glycine-low standards, I created a calibration curve by plotting the measured isotopic values vs. the known isotopic values relative to VPDB for carbon and N_2 for nitrogen. Using the calibration curve, measured isotopic values for samples can be calculated relative to the global standards. The measured isotopic value of glycine mid is also adjusted using the glycine calibration curve and compared to the known value relative to the global standards as an analysis check.

Table 2.1. Naming convention for the media and cell cultures from each of the growth conditions. Names are abbreviated as: carbon source nitrogen source / (conditions) / cell type. For example: pyruvate ammonium/yeast / wildtype = PyA/Y/WT.

Media Name	Cell Name	Carbon Source	Nitrogen Source	Other conditions	WT/Mutant
PyG	PyG/WT	Pyruvate (Py)	Glutamine (G)		WT
AcG	AcG/WT	Acetate (Ac)	Glutamine (G)		WT
PyG[a]	PyG/CSKO	Pyruvate (Py)	Glutamine (G)		CSKO
AcG[b]	AcG/CSKO	Acetate (Ac)	Glutamine (G)		CSKO
PyN	PyN/WT	Pyruvate (Py)	N_2 (N)		WT
AcN	AcN/WT	Acetate (Ac)	N_2 (N)		WT
PyA	PyA/WT	Pyruvate (Py)	Ammonium (A)		WT
AcA	AcA/WT	Acetate (Ac)	Ammonium (A)		WT
PyA/Y	PyA/Y/WT	Pyruvate (Py)	Ammonium (A)	Yeast (Y)	WT
AcA/Y	AcA/Y/WT	Acetate (Ac)	Ammonium (A)	Yeast (Y)	WT
PyA/YD	PyA/YD/WT	Pyruvate (Py)	Ammonium (A)	Yeast (Y), Dark (D)	WT
AcA/YD*	AcA/YD/WT*	Acetate (Ac)	Ammonium (A)	Yeast (Y), Dark (D)	WT

[a]Uses same media as row one. [b]Uses same media as row two. *Media not made, and cells not grown.

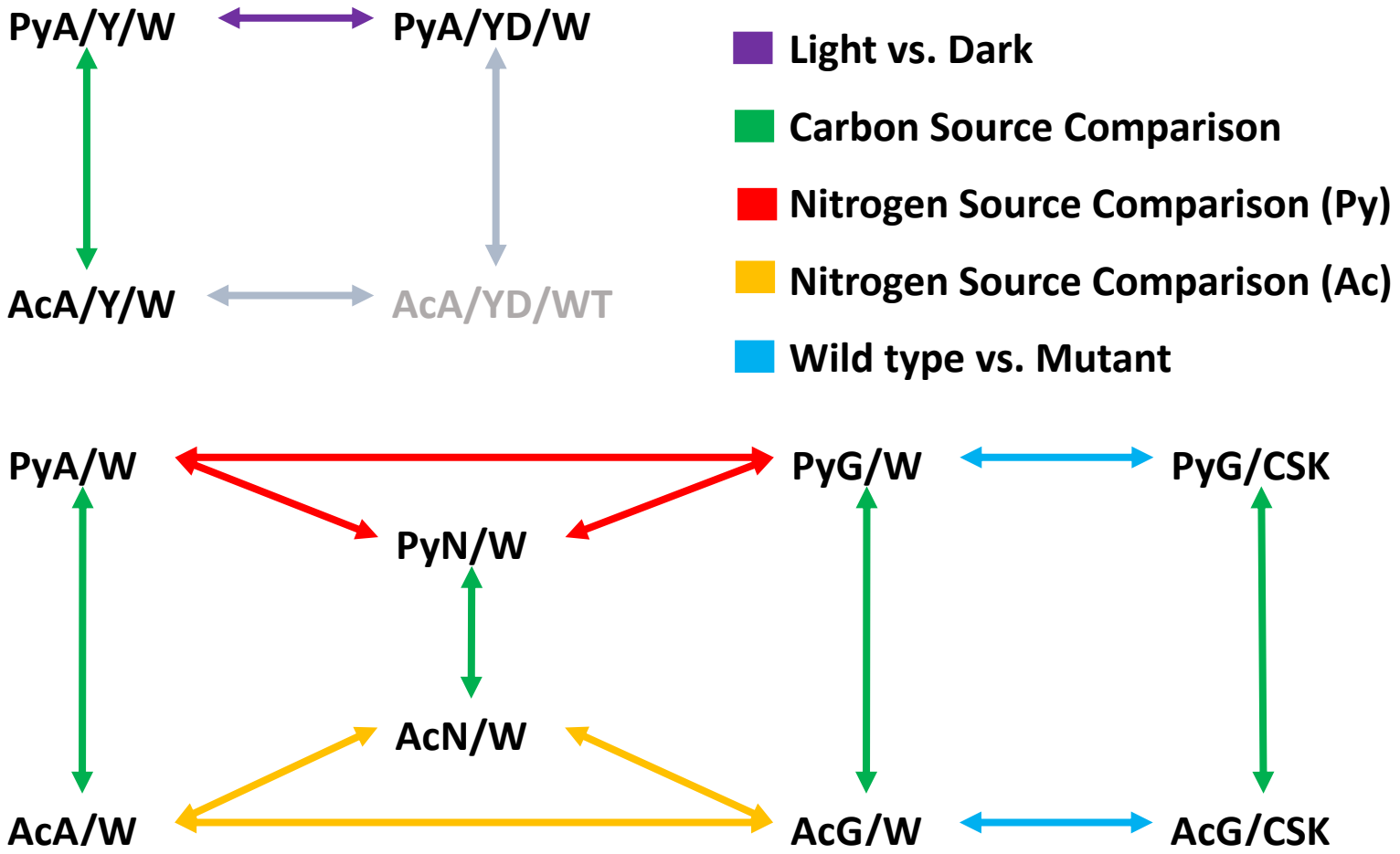

Figure 2.1. Cell culture relationships based on growth condition changes. Letter codes are detailed in Table 2.1. Arrows show the possible direct comparisons between cell cultures. Purple indicates a change in the energy source. Green indicates a change in the carbon source. Red and yellow indicate a change in the nitrogen source. Blue indicates the comparison between wild type and mutant cells. Grayed out text and arrows are a cell culture that was not grown.

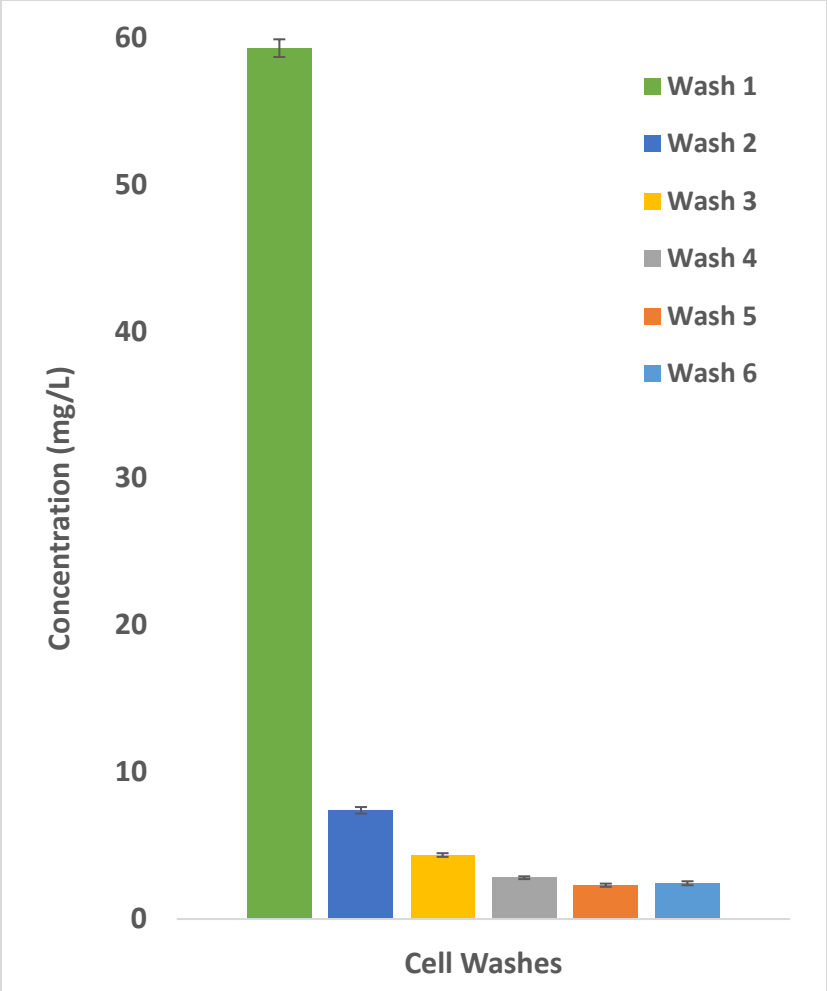

Figure 2.2. The carbon concentration for each of the supernatants collected after each wash step. The error shown is the standard deviation of three replicate measurements.

CHAPTER 3

RESULTS

3.1 Content and Composition Data for Media

The carbon and nitrogen content and isotopic compositions for each sample are expressed as the average of replicate samples (usually 3 to 5). Carbon or nitrogen content is expressed as mg C or mg N/mg dried material * 100, i.e., wt %. The error on each measurement is presented as the standard deviation of the replicate measurements. Carbon and nitrogen isotopic compositions are expressed in delta notation, i.e., $\delta^{13}C$ and $\delta^{15}N$ in units of per mil (‰) relative to their respective standards. The error for isotopic measurements is presented as the larger of the standard deviation of replicate measurements or the instrumental uncertainty (+/- 0.2 ‰).

To assess mass balance and interpret differences between the isotopic compositions of the cells and their various carbon and nitrogen sources, the carbon and nitrogen containing compounds in the media (i.e., sodium pyruvate, sodium acetate, ammonium chloride, glutamine, yeast extract, biotin, and EDTA) were measured individually (Table 3.1). The same stock bottles for each compound were used to make all versions of the media, except for acetate. Due to the bottle running out of material, acetate from two different bottles were used and are noted acetate-1 and acetate-2, respectively. Three compounds (acetate-2, glutamine, and ammonium chloride) were measured in two different ways. The first is measuring the compound straight from the

bottle. The second, marked with a prime (‘) measured the compound after it had been dissolved and lyophilized to mimic the method for measuring the media. The carbon isotope composition of acetate-2 and acetate-2’ were identical, with a $\delta^{13}C$ value of -31.84 ± 0.20 ‰. Acetate-1 was very different and had a $\delta^{13}C$ value of -14.23 ± 0.20 ‰. There is no nitrogen in acetate. Ammonium and ammonium’ had identical values for carbon and nitrogen content and isotopic values. The isotopic values for glutamine and glutamine’ were identical for $\delta^{13}C$ and $\delta^{15}N$, however, the carbon and nitrogen contents are not identical. Glutamine’ has a lower content for both carbon and nitrogen. The lower values could possibly be due to the lyophilization not being done for a long enough time, resulting in water accounting for extra weight in the sample.

In total, carbon and nitrogen data for eight different types of media were collected (Table 3.2). The first media used to grow cells was PyA/Y. I used this media to grow cells in the light and the dark. The other yeast containing media was AcA/Y. Yeast was originally used because *H. modesticaldum* cells grow much better when it is present; however, due to fact that the exact composition of yeast extract is unknown, the data is hard to interpret. In particular, it is not clear if the cells use yeast as a carbon and nitrogen source and, unfortunately, the yeast has the same isotopic composition as the pyruvate we used making it difficult to assess changes due to carbon source. Yeast was removed and the minimal salts media: PyA and AcA were used to grow all further cells. The AcA media was found to lose ammonium acetate nearly quantitatively due to volatilization during the lyophilization process. This also makes the results from these experiments hard to interpret. In this case, the data for the cells are likely fine, but the media is very

uncertain. To eliminate this volatilization loss, glutamine was substituted as the nitrogen source. This was beneficial as both the wild type and mutant cell cultures could grow on PyG and AcG media. The last pair of media that I used were PyN and AcN. These media did not contain ammonium or glutamine as a nitrogen source, forcing the cells to fix N_2 to obtain nitrogen.

In general, all media had carbon contents that ranged from ~6 to ~26 wt % carbon by weight. The PyA/Y media had the highest carbon content at 26.19 ± 0.07 wt % because of the addition of carbon-rich yeast. AcA/Y media had a carbon content of 24.07 ± 0.44 wt %. The PyA media had a carbon content of 13.80 ± 0.08 wt %. For the AcA media, carbon was lost during the lyophilization because the media contained acetate and ammonium. This loss of carbon resulted in AcA media having the lowest carbon content at 6.88 ± 0.21 wt %. PyG media had a carbon content of 21.73 ± 0.32 wt %. The AcG media had a carbon content of 16.28 ± 1.49 we %. The PyN media had a carbon content of 17.17 ± 0.27 wt %. The AcN media had a carbon content of 9.75 ± 1.22 wt %.

In general, all media had nitrogen contents that ranged from ~1.5 to ~8.5 wt % nitrogen by weight. The PyA/Y media had the highest nitrogen content at 8.47 ± 0.01 wt % because of the addition of yeast. The AcA/Y media has a lower nitrogen content than the PyA/Y media because AcA/Y media contains ammonium and acetate, leading to a loss of nitrogen during lyophilization. The AcA/Y had a nitrogen content of 7.36 ± 0.05 wt %. The PyA media had a nitrogen content of 5.53 ± 0.06 wt %. For the AcA media, nitrogen was lost during the lyophilization because the media contained acetate and ammonium. This loss of carbon resulted in AcA having a nitrogen content of only 1.55 ±

0.18 wt %. There was a significant loss of nitrogen due to removal of ammonium acetate during lyophilization. The PyG media had a nitrogen content of 4.68 ± 0.17 wt %. The AcG media had a nitrogen content of 4.84 ± 0.31 wt %. The nitrogen content for PyN and AcN were essentially below the quantitation limit for the calibration curve because there was no dissolved nitrogen in the media. What nitrogen may have been detected would be due to trace components from EDTA and biotin, or from N_2 gas equilibrating with the liquid media.

Media had a wide range of $\delta^{13}C$ values due to the big isotopic differences between the three abundant carbon containing compounds (sodium pyruvate, sodium acetate-1 and sodium acetate-2). The media ranged from about -32 to about -1.5 ‰. The PyA/Y media had a $\delta^{13}C$ of -24.88 ± 0.20 ‰. The AcA/Y media had a $\delta^{13}C$ of -22.20 ± 0.28 ‰. The PyA media had a $\delta^{13}C$ of -25.84 ± 0.20 ‰. The AcA media had the highest $\delta^{13}C$ at -1.34 ± 0.20 ‰. Due to the AcA media containing ammonium and acetate, the AcA media lost some carbon during lyophilization. This loss of carbon fractionated the carbon isotopes, resulting in an isotopically heavy sample. The PyG media had a $\delta^{13}C$ of -20.08 ± 0.20 ‰. The AcG media has relatively isotopically light acetate, but also has relatively isotopically heavy glutamine, resulting in a $\delta^{13}C$ of -19.02 ± 0.81 ‰. The PyN media had a $\delta^{13}C$ of -25.71 ± 0.20 ‰. The AcN media had the lowest $\delta^{13}C$ at -32.22 ± 0.20 ‰. This is because the media contains the isotopically light acetate-2 as a carbon source, while also not containing the isotopically heavier glutamine.

Media had a wide range of $\delta^{15}N$ values from about -1.5 to about 35 ‰. The PyA/Y media had a $\delta^{15}N$ value of 0.48 ± 0.23 ‰. The AcA/Y media had a $\delta^{15}N$ value of

4.70 ± 0.20 ‰. I have concluded that removal of ammonium acetate during the lyophilization of the media resulted in a large fractionation of the ammonium. The PyA media had the lowest $\delta^{15}N$ value at -1.34 ± 0.20 ‰. The AcA media had the highest $\delta^{15}N$ value at 34.78 ± 1.69 ‰. The outlier $\delta^{15}N$ value for AcA media is driven by the fractionation of nitrogen during the loss of ammonium during fractionization. The loss of ammonium is because of the presence of acetate and ammonium in the AcA media. The PyG media had a $\delta^{15}N$ value of -0.32 ± 0.65 ‰. The AcG media had a $\delta^{15}N$ value of 2.41 ± 0.92 ‰. The $\delta^{15}N$ values for PyN and AcN were highly uncertain because there was no abundant nitrogen source. What nitrogen may have been detected would be due to trace components from EDTA and biotin, or from N_2 gas equilibrating with the liquid media.

3.2 Content and Composition Data for Cell Cultures

Carbon and Nitrogen data were obtained for eleven cell cultures grown using the eight different media (Table 3.3). The PyA/Y/WT and PyA/YD/WT cells were both grown using the same media. For the two media that contained glutamine as the nitrogen source (PyG, AcG), both wild type and mutant cells were grown. The remaining five media each had only one corresponding cell culture (AcA/Y/WT, PyA/WT, AcA/WT, PyN/WT, and AcN/WT).

In general, all cells had carbon contents that ranged from ~47 to ~40 % carbon by weight. Interestingly, the PyA/YD/WT cells had the highest carbon content at 47.34 ± 0.46 %. The PyA/Y/WT cells had a carbon content of 43.46 ± 0.20 %. The AcA/Y/WT cells had a carbon content of 43.77 ± 0.19 %. The PyA/WT cells had a carbon content of

42.45 ± 0.35 %. The AcA/WT cells had a carbon content of 42.91 ± 0.72 %. The PyG/WT cells had a carbon content of 42.25 ± 0.26 %. The AcG/WT cells had a carbon content of 43.27 ± 1.84 %. The mutant cells grown in both growth conditions had higher carbon content than the wild type cells. The PyG/CSKO mutant cells had a carbon content of 44.22 ± 0.36 %. The AcG/CSKO mutant cells had a carbon content of 43.96 ± 0.14 %. The nitrogen fixing cells had the lowest carbon content values. The PyN/WT cells had a carbon content of 42.28 ± 0.03 %. The AcN/WT cells had the lowest carbon content at 39.63 ± 0.15 %.

In general, all cells had nitrogen contents that ranged from ~14 to ~11 % nitrogen by weight. The PyA/YD/WT cells had a nitrogen content of 14.19 ± 0.14 %. The PyA/Y/WT cells had a nitrogen content of 13.01 ± 0.2 %. The AcA/Y/WT cells had a nitrogen content of 12.22 ± 0.07 %. The PyA/WT cells had a nitrogen content of 12.51 ± 0.10 %. The AcA/WT cells had a nitrogen content of 12.19 ± 0.21 %. The PyG/WT cells had a nitrogen content of 12.84 ± 0.04 %. The AcG/WT cells had a nitrogen content of 12.33 ± 0.56 %. The mutant cells grown in both growth conditions had higher nitrogen content than the wild type cells. The PyG/CSKO mutant cells had a nitrogen content of 13.29 ± 0.09 %. The AcG/CSKO mutant cells had a nitrogen content of 12.47 ± 0.05 %. Like for the carbon content data, the nitrogen fixing cells had among the lowest nitrogen content. The PyN/WT cells had a nitrogen content of 12.20 ± 0.01 %. The AcN/WT cells had a nitrogen content of 10.68 ± 0.07 %.

In general, all media had $\delta^{13}C$ values that ranged from about -32 to about -18 ‰. The PyA/YD/WT cells had a carbon isotopic value of -25.60 ± 0.2 ‰. The PyA/Y/WT

cells had a $\delta^{13}C$ of -25.54 ± 0.2 ‰. The AcA/Y/WT cells had a $\delta^{13}C$ of -23.15 ± 0.20 ‰. The PyA/WT cells had a $\delta^{13}C$ of -25.48 ± 0.20 ‰. The AcA/WT cells had the lowest and only carbon isotopic value below -20 ‰ with a $\delta^{13}C$ -18.83 ± 0.20 ‰. The PyG/WT cells had a $\delta^{13}C$ of -27.12 ± 0.20 ‰. The AcG/WT cells had a $\delta^{13}C$ of -27.68 ± 0.20 ‰. The PyG/CSKO mutant cells had a $\delta^{13}C$ of -26.69 ± 0.20 ‰. The AcG/CSKO mutant cells had a $\delta^{13}C$ of -25.18 ± 0.20 ‰. The PyN/WT cells had a $\delta^{13}C$ of -22.22 ± 0.20 ‰. The AcN/WT cells had the and only carbon isotopic value above -30 ‰ with an $\delta^{13}C$ of -32.34 ± 0.30 ‰.

In general, all media had $\delta^{15}N$ that ranged from about -15 to about -4 ‰. The PyA/YD/WT cells had a $\delta^{15}N$ of -5.58 ± 0.61 ‰. The PyA/Y/WT cells had a $\delta^{15}N$ of -7.18 ± 0.2 ‰. The AcA/Y/WT cells had a $\delta^{15}N$ of -5.34 ± 0.20 ‰. The cells that grew in media without yeast and with ammonium as the nitrogen source had the lowest $\delta^{15}N$ values. The PyA/WT cells had a $\delta^{15}N$ of -13.31 ± 0.20 ‰. The AcA/WT cells had a $\delta^{15}N$ of -15.00 ± 0.20 ‰. The PyG/WT cells had $\delta^{15}N$ of -10.06 ± 0.20 ‰. The AcG/WT cells had a $\delta^{15}N$ of -6.05 ± 0.20 ‰. The PyG/CSKO mutant cells had a $\delta^{15}N$ of -8.91 ± 0.20 ‰. The AcG/CSKO mutant cells had a $\delta^{15}N$ of -4.59 ± 0.20 ‰. The cells that fixed nitrogen were nearly identical and had the highest $\delta^{15}N$ values. The PyN/WT cells had the lowest $\delta^{15}N$ value at -3.96 ± 0.20 ‰ The AcN/WT cells had a $\delta^{15}N$ of -4.04 ± 0.20 ‰.

Table 3.1. Carbon and nitrogen content and isotopic composition data for the components of the grown media. Values are presented as the mean of 3-5 replicate measurements. Uncertainty for the content data is the standard deviation of replicate measurements. Uncertainty for isotopic data is the higher value of either the standard deviation of replicate measurements or the instrumental uncertainty of 0.20 ‰. A prime (‘) indicates a compound that was dissolved and lyophilized prior to measurement.

Media Component	C wt % (mg/mg dw)	N wt % (mg/mg dw)	$\delta^{13}C$ (‰)	$\delta^{15}N$ (‰)
Sodium Pyruvate	31.66 ± 0.20	-	-25.82 ± 0.20*	-
Sodium Acetate-1	25.76 ± 0.35	-	-14.23 ± 0.20*	-
Sodium Acetate-2	28.09 ± 0.42	-	-31.84 ± 0.20*	-
Sodium Acetate-2’	27.27 ± 0.34	-	-31.85 ± 0.20*	-
Glutamine	39.15 ± 0.87	20.39 ± 0.28	-12.79 ± 0.31	-2.47 ± 0.20*
Glutamine’	30.44 ± 16.03	15.7 ± 8.09	-13.09 ± 0.28	-2.33 ± 0.20*
Ammonium Chloride	-	27.26 ± 0.20	-	-1.17 ± 0.20*
Ammonium Chloride’	-	27.92 ± 0.20	-	-1.23 ± 0.20*
Yeast Extract	39.19 ± 0.20	11.78 ± 0.20	-24.71 ± 0.20*	-1.49 ± 0.20*
Sodium Bicarbonate	13.28 ± 0.59	-	-1.86 ± 0.20*	-
EDTA	30.5 ± 0.20	7.48 ± 0.20	-25.93 ± 0.20*	-0.81 ± 0.20*
Biotin	48.9 ± 0.20	12.22 ± 0.20	-29.12 ± 0.32	3.35 ± 0.20*

*Uncertainty listed is instrumental uncertainty because it is higher than the standard deviation.

Table 3.2. Carbon and nitrogen content and isotopic composition data for eight media samples. Values are presented as the mean of 3-5 replicate measurements. Uncertainty for content data is the standard deviation of replicate measurements. Uncertainty for isotopic is the higher value of either the standard deviation of replicate measurements or the instrumental uncertainty of 0.20 ‰.

Media Type	C wt % (mg/mg dw)	N wt % (mg/mg dw)	$\delta^{13}C$ (‰)	$\delta^{15}N$ (‰)
PyA/Y	26.19 ± 0.20	8.47 ± 0.20	-24.87 ± 0.20*	0.48 ± 0.23
AcA/Y	24.07 ± 0.44	7.36 ± 0.20	-22.2 ± 0.28	4.7 ± 0.20*
PyA	13.8 ± 0.20	5.53 ± 0.20	-25.84 ± 0.20*	-1.34 ± 0.20*
AcA	6.88 ± 0.21	1.55 ± 0.20	-14.54 ± 0.3	34.78 ± 1.69
PyG	21.73 ± 0.32	4.68 ± 0.17	-20.08 ± 0.20*	-0.32 ± 0.65
AcG	16.28 ± 1.49	4.84 ± 0.31	-19.02 ± 0.81	2.41 ± 0.92
PyN	17.17 ± 0.27	0.05 ± 0.20	-25.71 ± 0.20*	-3.49 ± 3.59
AcN	9.75 ± 1.22	0.06 ± 0.20	-32.22 ± 0.20*	-0.02 ± 1.98

*Uncertainty listed is instrumental uncertainty because it is higher than the standard deviation.

Table 3.3. Carbon and nitrogen content and isotopic composition data for eleven cell samples. Values are presented as the mean of 3-5 replicate measurements. Uncertainty for content data is the standard deviation of replicate measurements. Uncertainty for isotopic is the higher value of either the standard deviation of replicate measurements or the instrumental uncertainty of 0.20 ‰.

Cells	% C (mg/mg dw)	% N (mg/mg dw)	$\delta^{13}C$ (‰)	$\delta^{15}N$ (‰)
PyA/Y/WT	43.46 ± 0.20	13.01 ± 0.20*	-25.54 ± 0.20*	-7.18 ± 0.20*
PyA/YD/WT	47.34 ± 0.46	14.19 ± 0.20*	-25.60 ± 0.24	-5.58 ± 0.61
AcA/Y/WT	43.77 ± 0.20	12.22 ± 0.20*	-23.15 ± 0.20*	-5.34 ± 0.20*
PyA/WT	42.45 ± 0.35	12.51 ± 0.20*	-25.48 ± 0.20*	-13.31 ± 0.20*
AcA/WT	42.91 ± 0.72	12.19 ± 0.21	-18.83 ± 0.20*	-15.00 ± 0.20*
PyG WT	42.25 ± 0.26	12.84 ± 0.04	-27.12 ± 0.20*	-10.06 ± 0.20*
AcG WT	43.27 ± 1.84	12.33 ± 0.56	-27.68 ± 0.20*	-6.05 ± 0.20*
PyG CSKO	44.22 ± 0.36	13.29 ± 0.09	-26.69 ± 0.20*	-8.91 ± 0.20*
AcG CSKO	43.96 ± 0.14	12.47 ± 0.05	-25.18 ± 0.20*	-4.59 ± 0.20*
PyN/WT	42.28 ± 0.20*	12.20 ± 0.20*	-22.22 ± 0.20*	-3.96 ± 0.20*
AcN/WT	39.63 ± 0.20*	10.68 ± 0.20*	-32.34 ± 0.30	-4.04 ± 0.20*

*Uncertainty listed is instrumental uncertainty because it is higher than the standard deviation

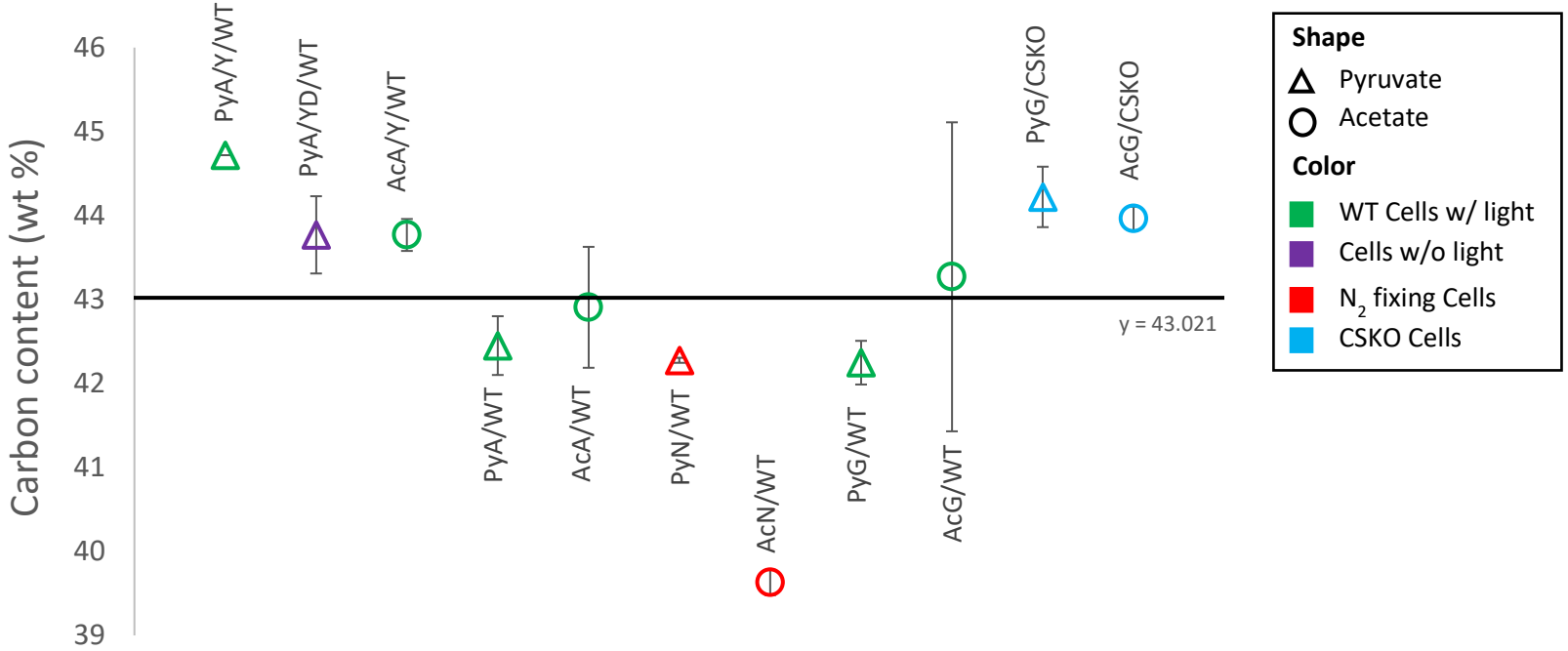

Figure 3.1. Carbon content data for all 11 cultures grown. Values are the carbon content of the cells with units of weight percent. Triangles indicate cells grown with pyruvate as a carbon source. Circles indicate cells grown with acetate as a carbon source. Purple indicates cells grown in the dark. Red indicates cells that were forced to use nitrogen fixation. Blue indicates cells that had the citrate synthase knockout mutation. Error bars indicate the standard deviation of replicate measurements.

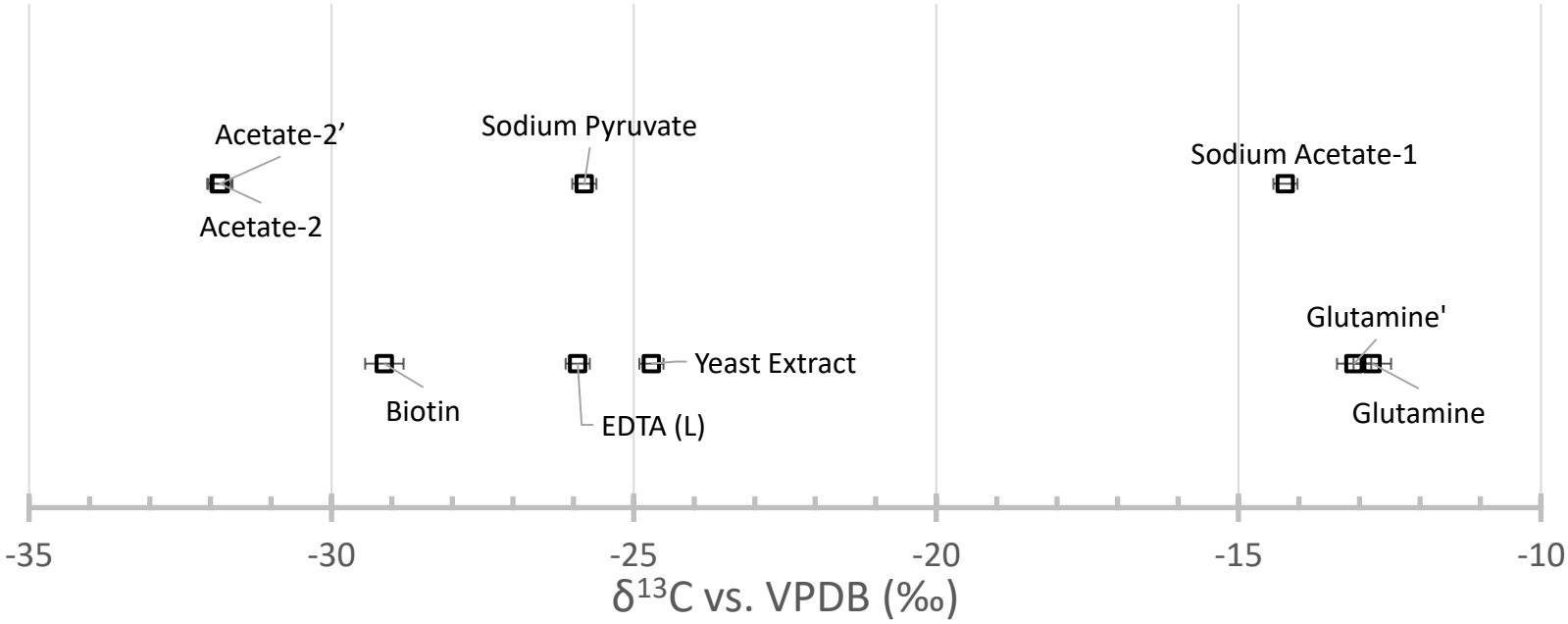

Figure 3.2. Carbon isotopic data for individual components of the media. The top row are major carbon sources in a media. Error bars indicate the uncertainty of the instrument (+/- 0.2 ‰) unless the standard deviation of replicate measurements resulted in a higher value.

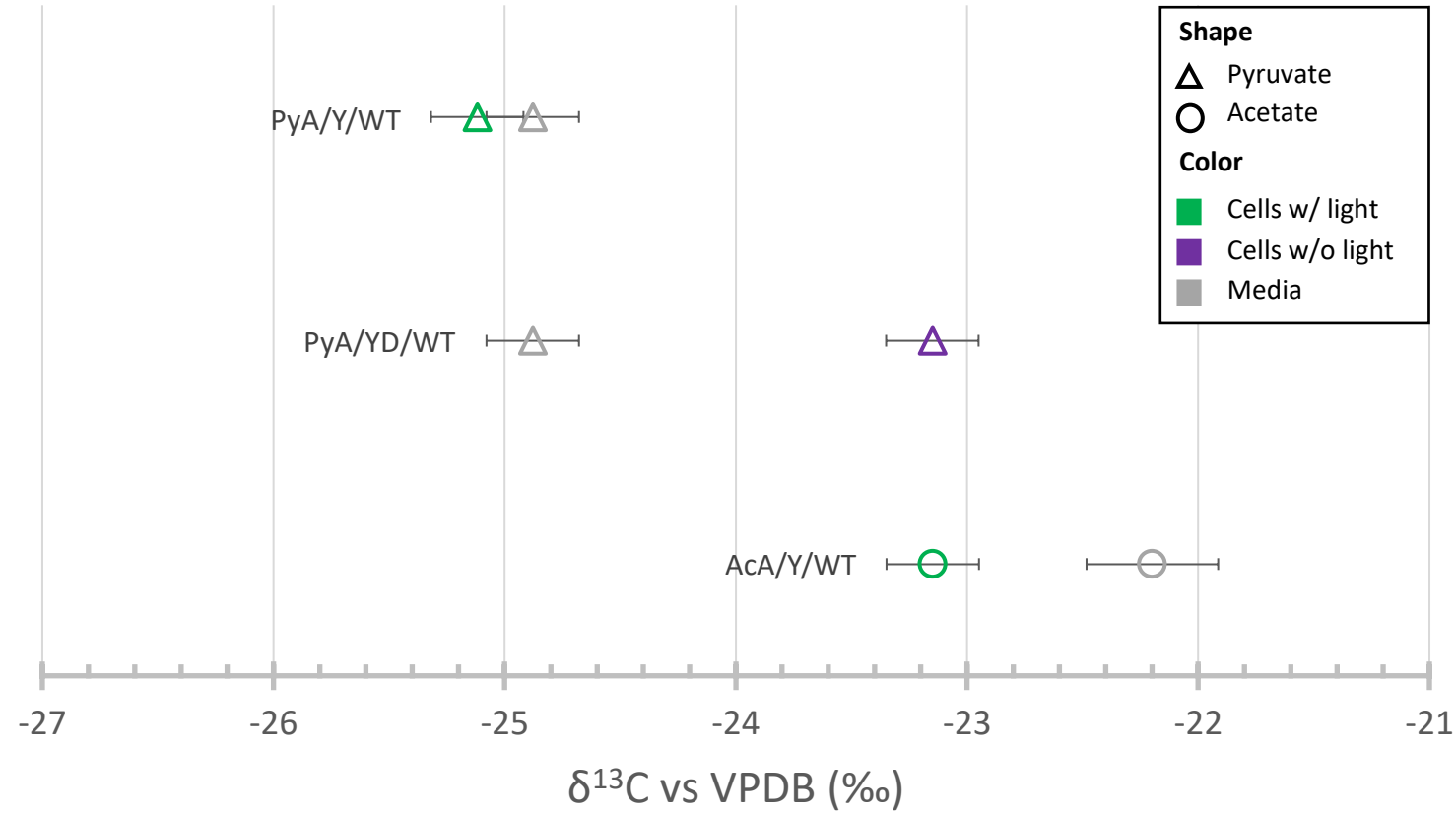

Figure 3.3. Carbon composition data for 3 cultures grown with yeast. Values are $\delta^{13}C$ values of the cells (green, purple) and media (grey) with units of ‰. Each row corresponds to the cells and media for one growth condition. Triangles indicate cells grown with pyruvate as a carbon source. Circles indicate cells grown with acetate as a carbon source. Purple indicates cells grown in the dark. Error bars indicate the uncertainty of the instrument (+/- 0.2 ‰) unless the standard deviation of replicate measurements resulted in a higher value.

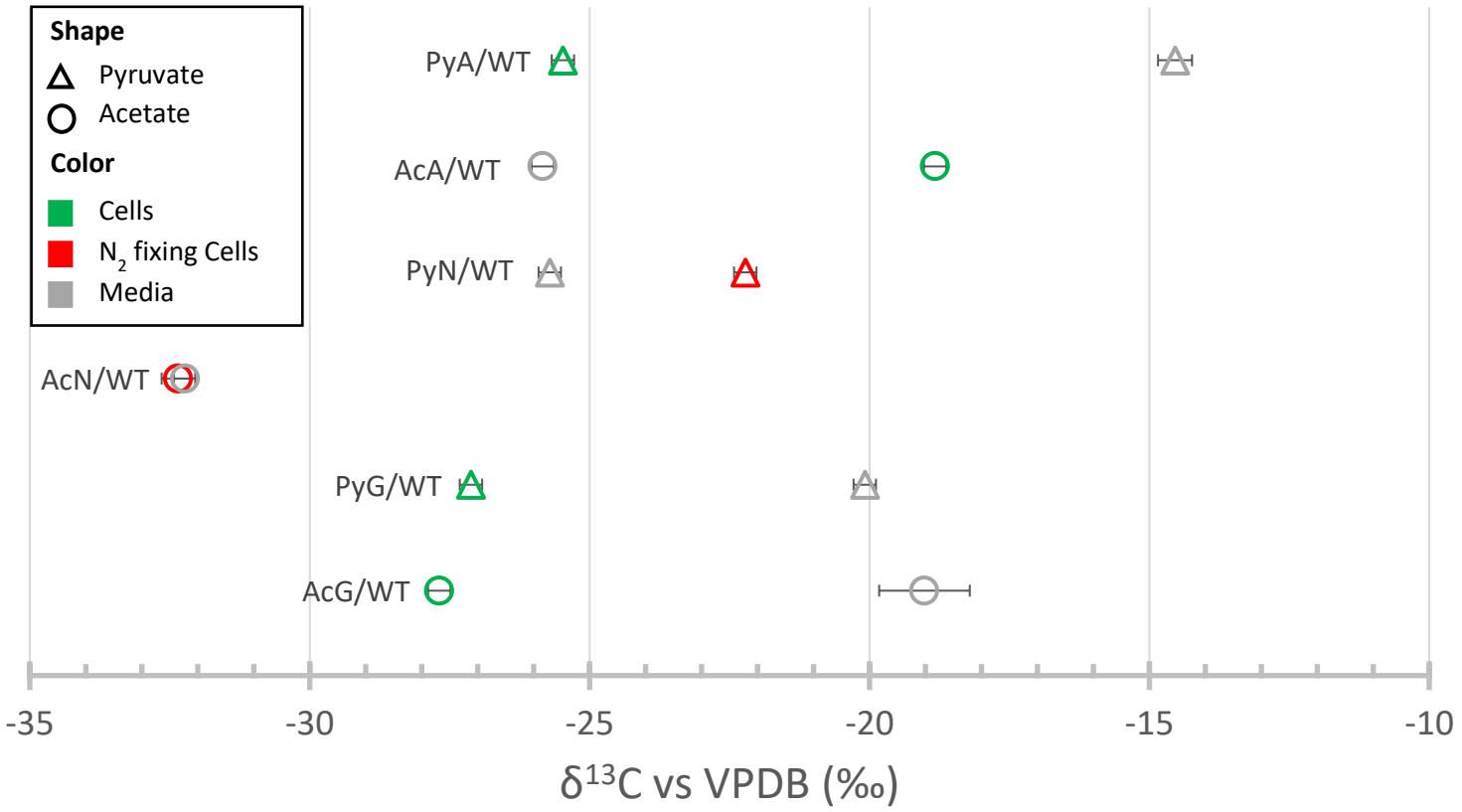

Figure 3.4. Carbon composition data for 6 cultures grown with three different nitrogen sources. Values are $\delta^{13}C$ values of the cells (green, red) and media (grey) with units of ‰. Each row corresponds to the cells and media for one growth condition. Triangles indicate cells grown with pyruvate as a carbon source. Circles indicate cells grown with acetate as a carbon source. Red indicates cells forced to fix nitrogen. Error bars indicate the uncertainty of the instrument (+/- 0.2 ‰) unless the standard deviation of replicate measurements resulted in a higher value.

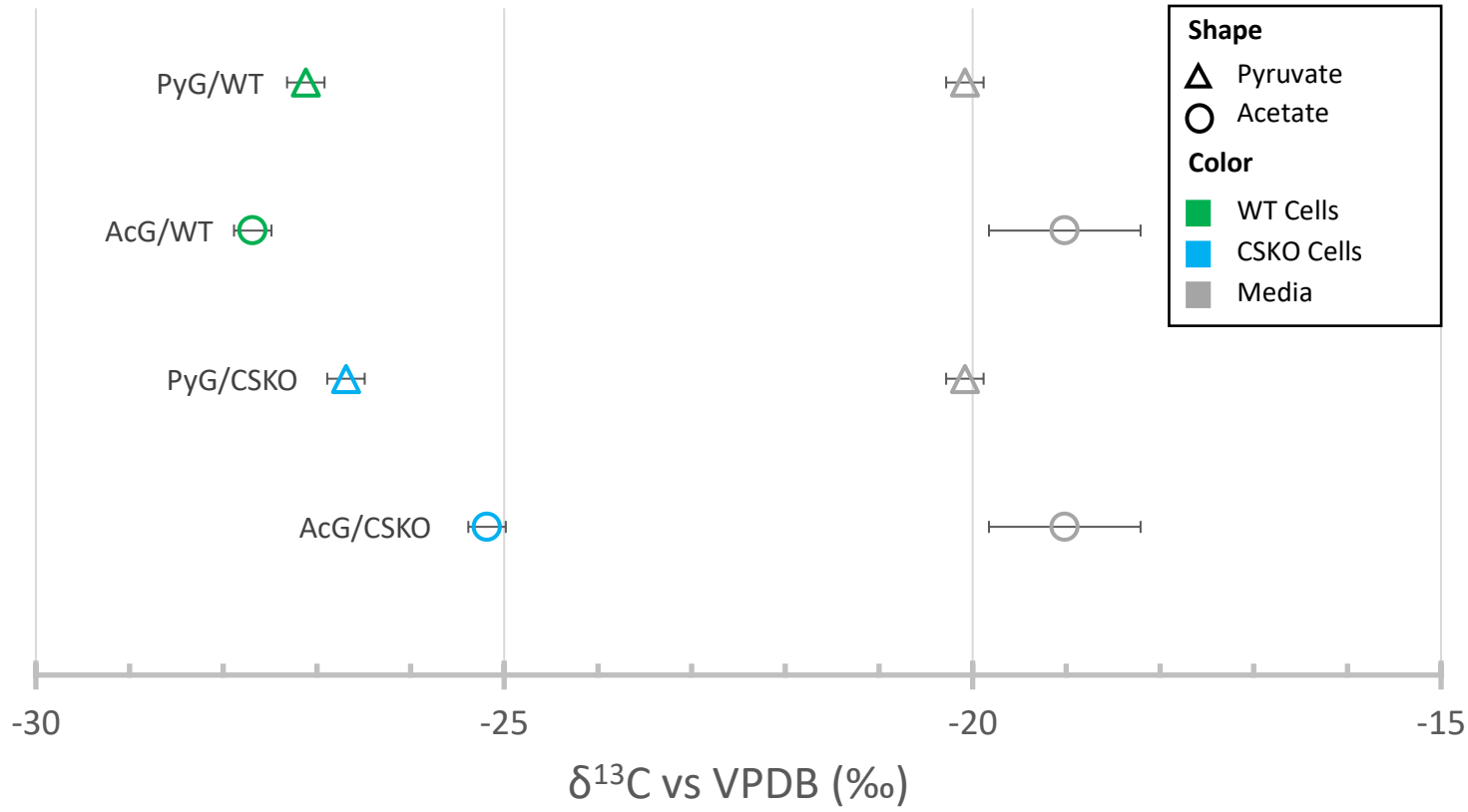

Figure 3.5. Carbon composition data for 2 wild type and 2 mutant cultures grown with glutamine. Values are $\delta^{13}C$ values of the cells (green, blue) and media (grey) with units of ‰. Each row corresponds to the cells and media for one growth condition. Triangles indicate cells grown with pyruvate as a carbon source. Circles indicate cells grown with acetate as a carbon source. Blue indicates mutant cells. Error bars indicate the uncertainty of the instrument (+/- 0.2 ‰) unless the standard deviation of replicate measurements resulted in a higher value.

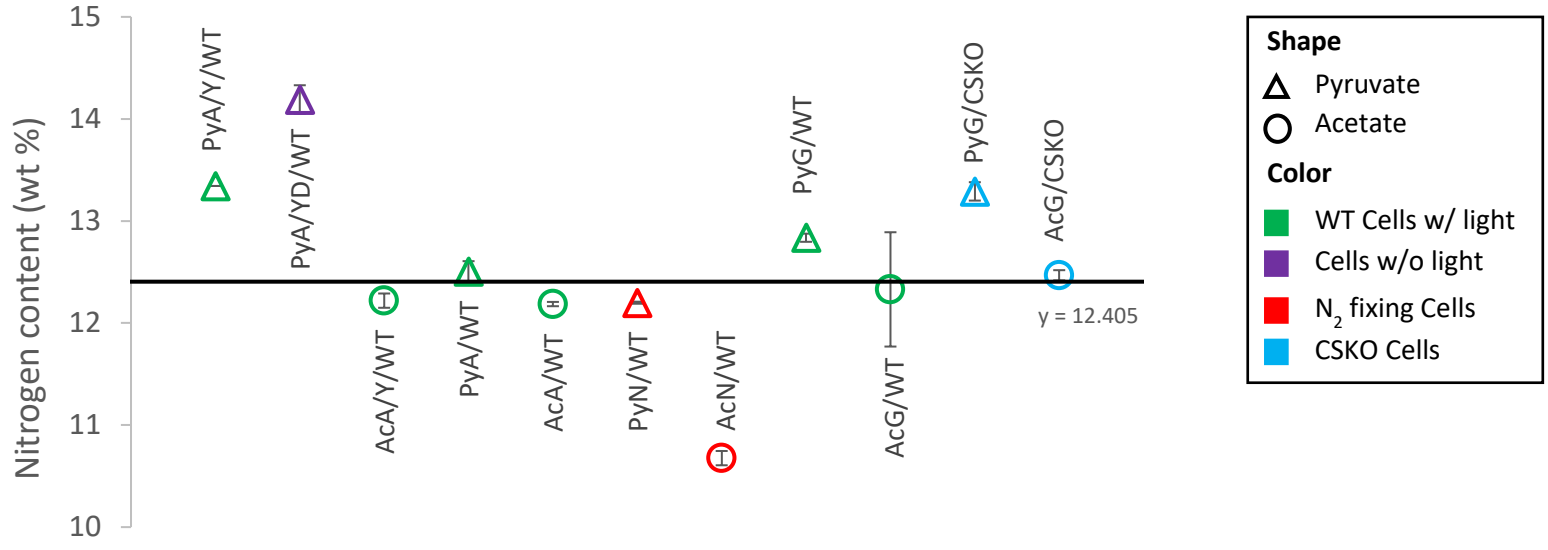

Figure 3.6. Nitrogen content data for all 11 cultures grown. Values are the nitrogen content of the cells with units of weight percent. Triangles indicate cells grown with pyruvate as a carbon source. Circles indicate cells grown with acetate as a carbon source. Purple indicates cells grown in the dark. Red indicates cells that were forced to use nitrogen fixation. Blue indicates cells that had the citrate synthase knockout mutation. Error bars indicate the standard deviation of replicate measurements.

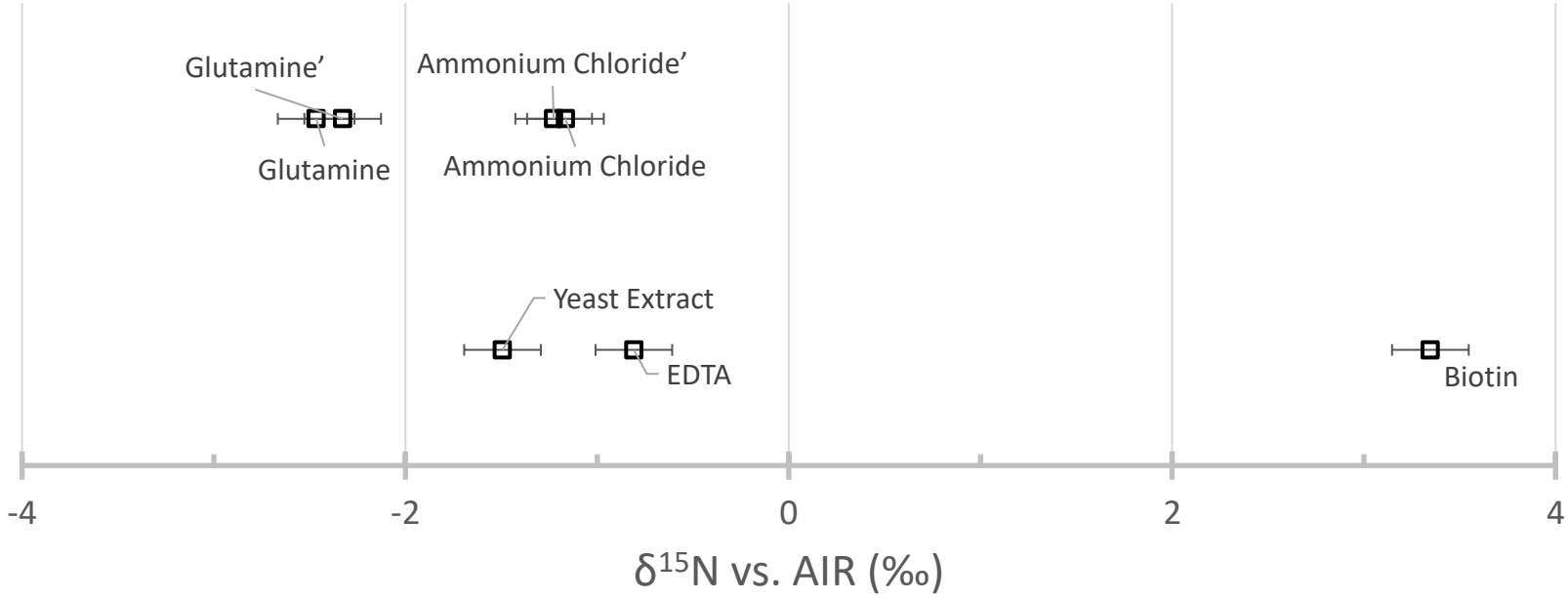

Figure 3.7. Nitrogen isotopic data for individual components of the media. Error bars indicate the uncertainty of the instrument unless the standard deviation of replicate measurements resulted in a higher value

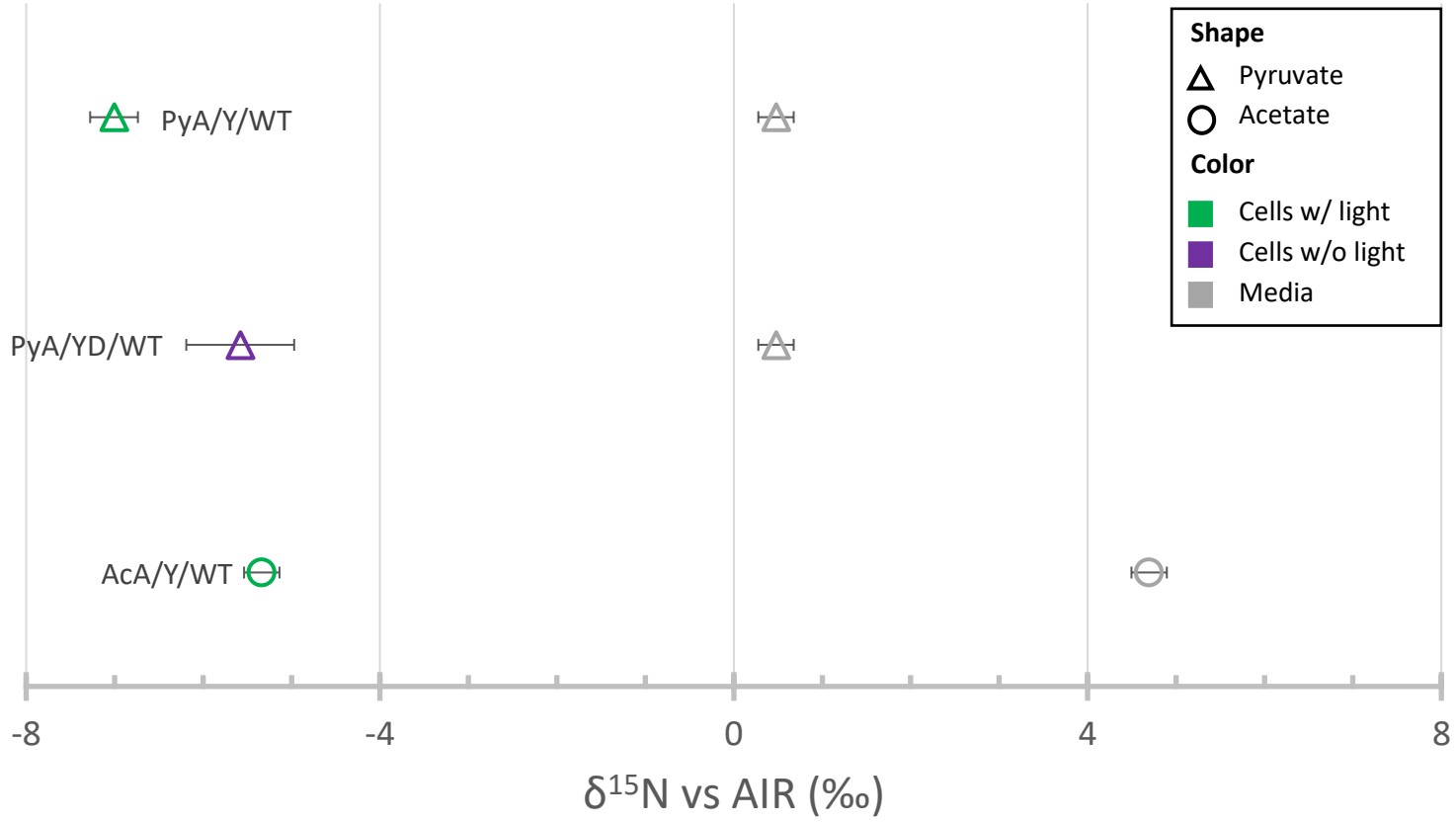

Figure 3.8. Nitrogen composition data cells grown on yeast. Values are $\delta^{15}N$ values of the cells (green, purple) and media (grey) with units of ‰. Each row corresponds to the cells and media for one growth condition. Triangles indicate cells grown with pyruvate as a carbon source. Circles indicate cells grown with acetate as a carbon source. Purple indicates cells grown in the dark. Error bars indicate the uncertainty of the instrument (+/- 0.2 ‰) unless the standard deviation of replicate measurements resulted in a higher value.

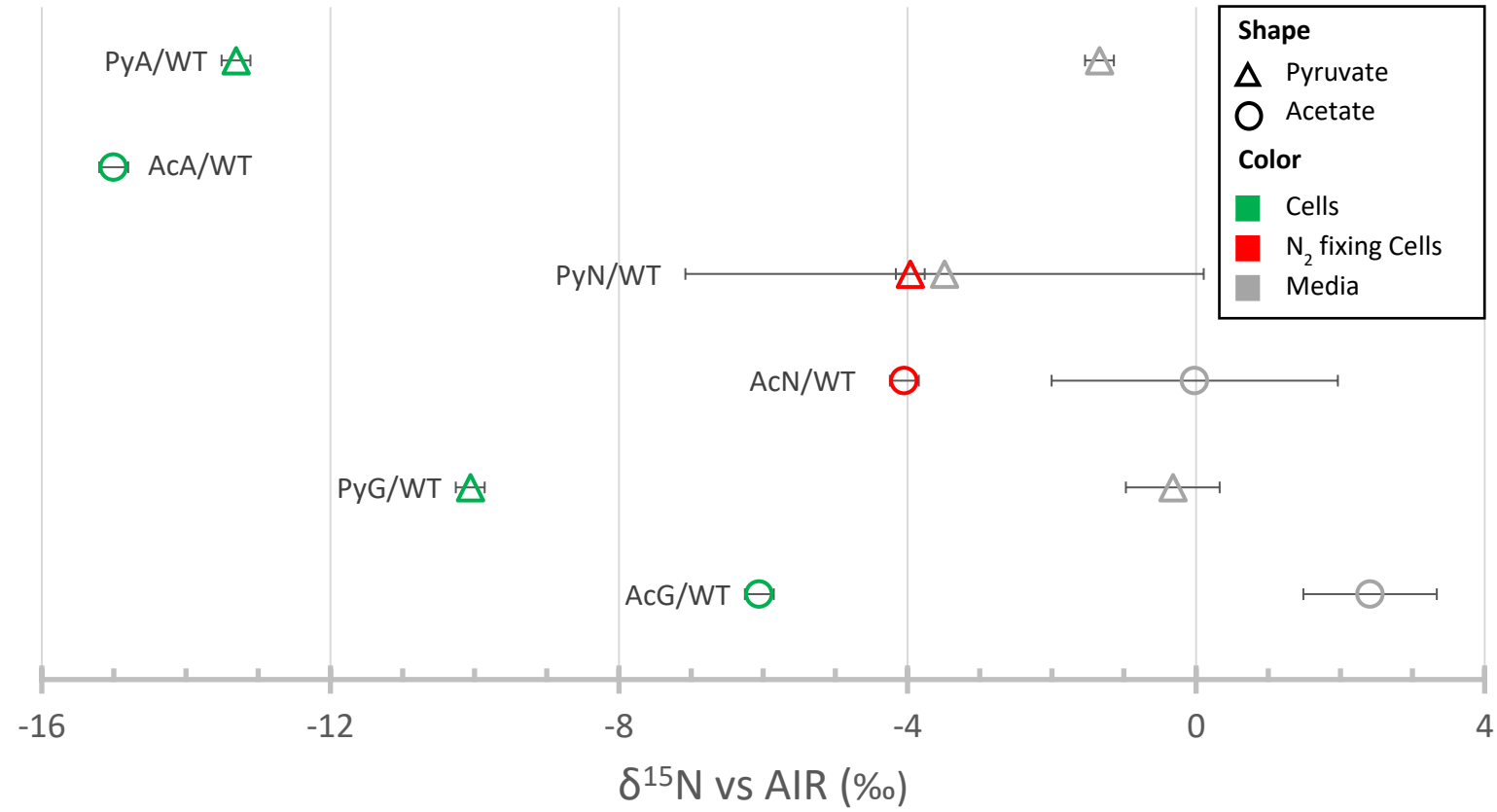

Figure 3.9. Nitrogen composition data for 6 cultures grown with three different nitrogen sources. Values are $\delta^{15}N$ values of the cells (green, red) and media (grey) with units of ‰. Each row corresponds to the cells and media for one growth condition. Triangles indicate cells grown with pyruvate as a carbon source. Circles indicate cells grown with acetate as a carbon source. Red indicates cells that were forced to fix nitrogen. Error bars indicate the uncertainty of the instrument (+/- 0.2 ‰) unless the standard deviation of replicate measurements resulted in a higher value.

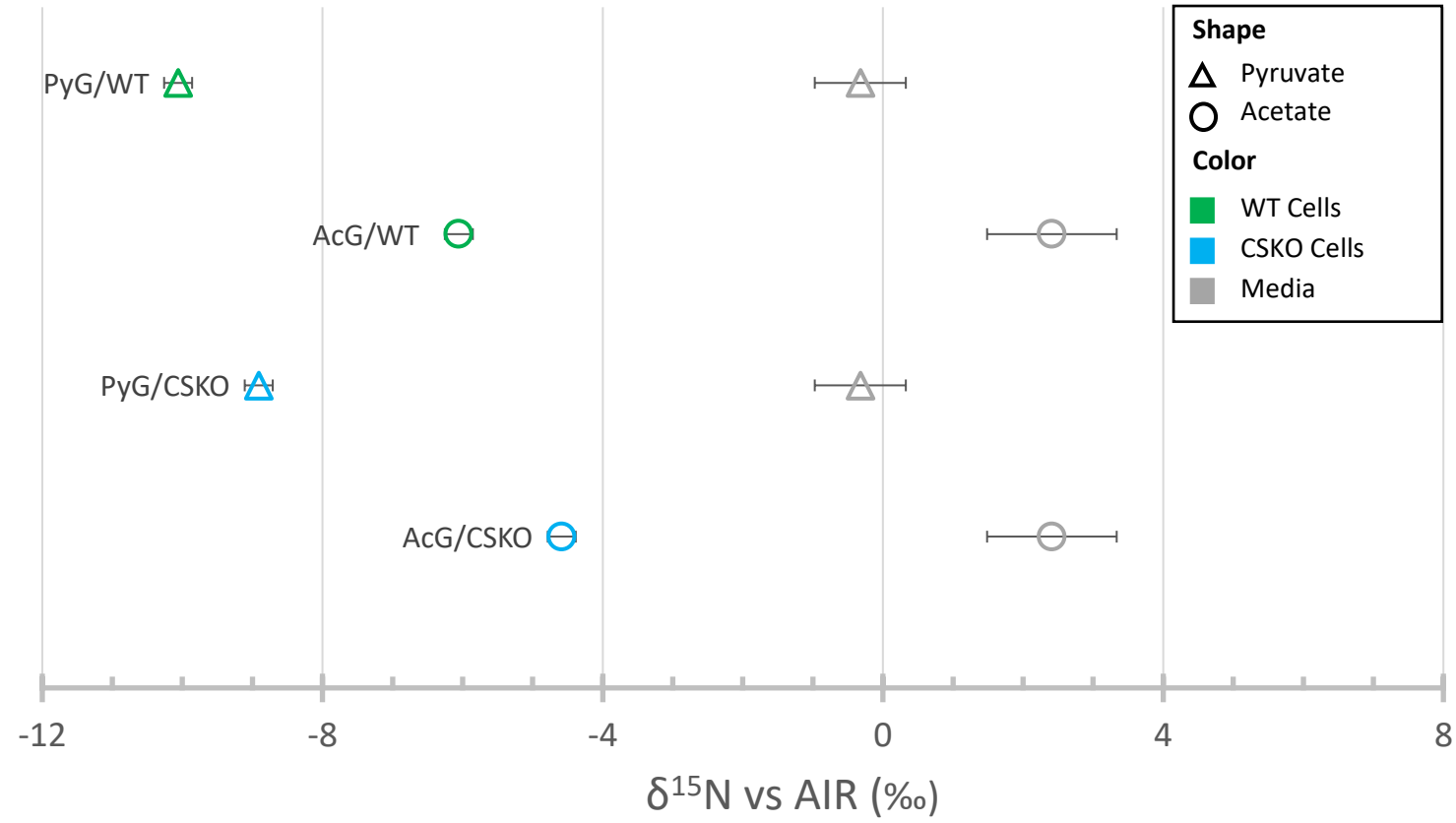

Figure 3.10. Nitrogen composition data for 2 wild type and 2 mutant cultures grown with glutamine. Values are $\delta^{15}N$ values of the cells (green, blue) and media (grey) with units of ‰. Each row corresponds to the cells and media for one growth condition. Triangles indicate cells grown with pyruvate as a carbon source. Circles indicate cells grown with acetate as a carbon source. Blue indicates mutant cells. Error bars indicate the uncertainty of the instrument (+/- 0.2 ‰) unless the standard deviation of replicate measurements resulted in a higher value.

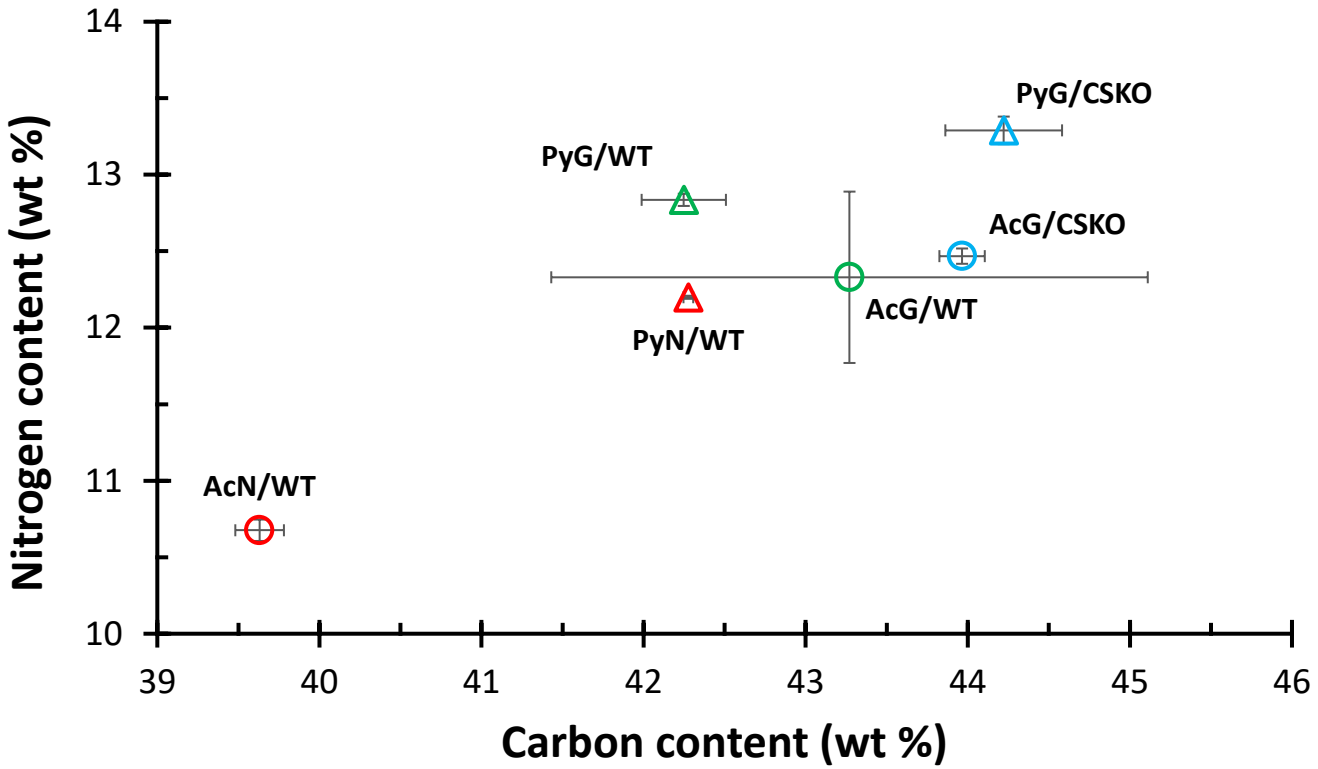

Figure 3.11. Carbon and nitrogen content data for six of the cell cultures: PyN/WT, AcN/WT, PyG/WT, AcG/WT, PyG/CSKO, AcG/CSKO. The error bars indicate the standard deviation of replicate measurements. Green points indicate wild type cells grown with glutamine as a nitrogen source. Red points indicate wild type cells that fixed nitrogen. Blue points indicate mutant cells. Triangles indicate cells grown with pyruvate as a carbon source. Circles indicate cells grown with acetate as a carbon source.

CHAPTER 4

DISCUSSION

4.1 Carbon Sources

Heliomicrobium modesticaldum can use light or chemical reactions as a source of energy, which means it can act as either a photoorganoheterotroph or chemoorganoheterotroph. To explore this difference, I grew H. modesticaldum in identical media with one culture exposed to light (PyA/Y/WT), and one culture placed in the dark (PyA/YD/WT). I expected the culture grown in the light to grow the best, and therefore, have the highest carbon content at about 50 wt % (Lawford and Rousseau, 1996). Light is a more abundant and less costly energy source. Growing as a chemotroph is more costly because the reaction used to generate energy is pyruvate fermentation. Bacteria that are growing on pyruvate as the carbon source and use pyruvate for pyruvate fermentation as the energy source use more of the pyruvate. By using pyruvate fermentation, the cells may exhibit a different isotopic signature due to the additional reaction. The cells grown in the light still use pyruvate fermentation, but as the amount of light increases, the amount of energy from pyruvate fermentation decreases. Both cultures are still heterotrophs and were grown on the same carbon source, so I expected their $\delta^{13}C$ to be similar to each other and to the media they were growing on (Blair et al., 1985; Zyakun et al., 2009).

I expected the nitrogen content and $\delta^{15}N$ for the two cell cultures to be equivalent because they were given the same reduced nitrogen source, and thus I was not expecting there to be a big difference in how nitrogen was incorporated into the cell. Cells in general have a rough nitrogen content of about 10 to 15 wt % (Lawford and Rousseau, 1996; Tchobanoglous et al., 2014). Cells that assimilate ammonium typically fractionate nitrogen isotopes by about -7 to -20 ‰ (Fogel and Cifuentes, 1993). The nitrogen components from the media have an isotopic value of about -1 ‰, so typical nitrogen fractionation during ammonium assimilation would put my cells in the range of -8 to -20 ‰. Due to my results being expressed as an average of replicate injections, I am considering a p-value of < 0.01 to be statistically different.

The $\delta^{13}C$ values were the only values that matched what I was expecting for the general pattern for heterotrophic cells (i.e., the cells looked like the culture media). Cells grown in both the light and the dark had a $\delta^{13}C$ of about -25.6 ‰, and the media had a $\delta^{13}C$ of -25 ‰. This indicates that the cells act as typical heterotrophs (Boschker and Middelburg, 2002) regardless of which energy source they are using. The carbon and nitrogen contents of the cells grown on pyruvate in the light (44.46 ± 0.20 wt % C, 13.01 ± 0.20 wt % N) were lower than the cells grown in the dark (47.34 ± 0.46 wt % C, 14.19 ± 0.14 wt % N). It is unclear why growing without a cheap and abundant energy source in the form of light would result in cells that were carbon and nitrogen poor compared to the cells that had to use pyruvate fermentation.

Contrary to my expectation, the $\delta^{15}N$ for the cells grown in the dark was higher (-5.58 ± 0.61 ‰) than the cells grown in the light (-7.18 ± 0.20 ‰). It is also not exactly

clear what may have happened here, and the presence of yeast in the media could cause issues with interpreting this data. The unknown makeup of yeast extract means that other compounds might have been present, or additional processes could have happened for which I cannot account. Yeast extract contains carbon and nitrogen, so we don't know the true carbon and nitrogen sources that influenced the cells. If compounds or processes influenced the content or isotopic composition for these cells, this data should be interpreted with caution. Similarly, the cells grown on acetate with yeast reflect their C and N sources ($\delta^{13}C$: 43.77 ± 0.20 ‰, $\delta^{15}N$: 12.22 ± 0.20 ‰), but have the same issues involving interpretation with yeast present.

To attempt to alleviate uncertainties due to the presence of yeast extract, all future cultures were grown on minimal salts media with no yeast extract. We note that cells did not grow on minimal media in the dark with acetate.

4.2 Nitrogen sources

Heliomicrobium modesticaldum is able to fix nitrogen (Asao and Madigan, 2010; Tang et al., 2010), but does not when given a readily available form of nitrogen such as ammonium. There is significant literature on nitrogen isotopes for both nitrogen fixation, which ranges from -3 ‰ to +1 ‰ for molybdenum-iron nitrogenase (Fogel and Cifuentes, 1993; Unkovich, 2013; Zhang et al., 2014), and nitrogen assimilation, which ranges from -6.5 ‰ to -20 ‰ (Fogel and Cifuentes, 1993; Lawford and Rousseau, 1996). The first nitrogen source I grew *H. modesticaldum* on was ammonium chloride. Due to the nitrogen source being the same as the previous growth conditions, I once again expected the cells to fall within the $\delta^{15}N$ range of -8 to -20 ‰ that is typical of ammonia

assimilation. I also expected the nitrogen content to be slightly less than when grown on yeast because a potential usable nitrogen source within the yeast was taken away. Ammonium was still readily available; thus, I did not expect the nitrogen content to be significantly changed. With light present, the cells grew as photoorganoheterotrophs. Therefore, I still expected their $\delta^{13}C$ value to reflect the carbon source they were consuming, as typically seen in heterotrophs (Boschker and Middelburg, 2002; Fogel and Cifuentes, 1993). Similar to the nitrogen content of the cells, I expected the carbon content to be lower than when the cells had access to yeast but still high, as they are not limited by nitrogen or carbon. The cells were grown on either pyruvate or acetate in the minimal media with ammonium. Without any yeast to provide unexpected nitrogen or carbon sources, the carbon content, nitrogen content, and $\delta^{15}N$ values of the cells grown on both carbon sources were expected to be equivalent. Due to the pyruvate and acetate-1 compounds having different $\delta^{13}C$ values, -25.82 ± 0.20 ‰ and -14.23 ± 0.20 ‰ respectively, I expected there to be a difference in $\delta^{13}C$ values for the cells. If each cell culture reflected their carbon source, cells grown on pyruvate would be in the -25 to -28 ‰ range, while the cell grown on acetate would be in the -12 ‰ to -14 ‰ range.

For both growth conditions, the carbon content and nitrogen content of the cells were near the average for all my cells. Both growth conditions produced cells with $\delta^{15}N$ values in the expected range. Cells grown on AcA media were lighter at -15.00 ‰ than cells grown on PyA media at -13.31 ± 0.20 ‰. These values are significantly different from those obtained when the cultures were grown with yeast present, providing evidence that the presence of yeast had an impact on the growth of the cells. The fraction of $\delta^{15}N$

from the media can only be measured accurately for cells grown on pyruvate. For the AcA media, the presence of ammonium and acetate during lyophilization results in the volatilization of ammonium acetate, which all but completely removes N from the media. Not only does this impact the measured nitrogen content values, but the process also strongly fractionates the nitrogen isotopes. The measured $\delta^{15}N$ for the media was +34.7 ‰. This large positive value reflects the near complete removal of nitrogen with a preference for the removal of light isotopes. Considering that the light isotopes are favored in the less condensed (volatile) phase, it makes sense that the heavy isotopes were the only ones that remained after volatilization.

The only major source of nitrogen in the AcA media was ammonium chloride with a $\delta^{15}N$ value of -1 ‰. The PyA media, in contrast, does not contain acetate, and ammonium pyruvate is not volatile. There was no loss of pyruvate or ammonium during lyophilization; the media had a $\delta^{15}N$ value of -1.34 ± 0.20 ‰, which is nearly identical to the ammonium chloride (p value). Moreover, nitrogen mass balance was obtained (e.g., the difference between the weighted isotopic composition of each of the media components and the value for the media was very small). Assuming the AcA media should have a similar $\delta^{15}N$ value to PyA due to both media having identical nitrogen containing compounds, the fractionation of nitrogen from the ammonium compound can be calculated (Table 4.1). For both growth conditions, fractionation of nitrogen during ammonium assimilation falls within the expected range (Fogel and Cifuentes, 1993; Hoch et al., 1994). The nitrogen content of the PyA/WT cells (12.51 ± 0.10 wt %) and

AcA/WT cells (12.19 ± 0.21 wt %) were within one standard deviation of the average nitrogen content for my cells at 12.40 wt % (Fig. 3.6).

The $\delta^{13}C$ of cells grown on pyruvate was -25.48 ± 0.20 ‰. With the pyruvate component having a $\delta^{13}C$ of -25.82 ± 0.20 ‰, the cells' $\delta^{13}C$ does reflect their carbon source. The same cannot be said of the cells grown on acetate. There was a significant difference between the cells and the acetate they were grown on ($\Delta_{cell\text{-}acetate}$ = -4.6 ‰). For carbon, the media did satisfy the isotopic mass balance, meaning the media had a similar $\delta^{13}C$ value and thus a similar value for $\Delta_{cell\text{-}acetate}$ (-4.29 ‰). Changing carbon sources does seem to have an impact on the carbon fractionation. The cells grown on acetate do not reflect their media, nor are they equivalent in $\delta^{13}C$ value to the cells grown on pyruvate. By forcing the cells to start their carbon metabolism with acetate, they are forced to perform an extra step to make their own pyruvate. The extra step requires the addition of CO_2 and acetate to make pyruvate. Assuming the cells behave the same until the point at which they acquire pyruvate (either from the media or making it), the observed difference in the isotopic values and the fractionation of carbon from the media can be attributed to one of two things: how the carbon source is brought into the cell from the media, or the reaction between acetate and CO_2 to create pyruvate.

The second nitrogen source that was used to grow *H. modesticaldum* was glutamine. Glutamine does contain carbon, but it is isotopically heavy (-13.09 ± 0.20 ‰) compared to all the cell cultures. I do not believe much glutamine was used as a carbon source by the cells, as they do not reflect the isotopic composition of the glutamine. In addition to testing a different nitrogen source, using glutamine also solves the problem of

ammonium volatilization during the lyophilization step of my sample preparation. Glutamine is different from ammonium in that it has two nitrogen per molecule. Both of these nitrogen are in a reduced form as NH_2-R, which should result in cells having similar $\delta^{15}N$ values (Fogel and Cifuentes, 1993). I expected the cells to have similar nitrogen content values to the cells grown on ammonium. For carbon isotope values, H. modesticaldum were still being grown as photoorganoheterotrophs, so I did not expect to see a change for cells grown on pyruvate. The cells grown on AcG media were the first to be grown on acetate-2. Due to the acetate-2 $\delta^{13}C$ being light compared to acetate-1, I was uncertain what the $\delta^{13}C$ of the cells would be, or if the cells would fractionate the carbon as they appeared to when grown on ammonium and acetate-1. I also did not expect the change of nitrogen source to affect the carbon content of the cells.

Growing cells in PyG and AcG media allowed me to make comparisons between these two cell cultures and to make comparisons with the cells grown on ammonium as the nitrogen source. I can also compare the values measured to what I expected based on my previous results and published literature. The $\delta^{15}N$ values for cells grown on PyG and AcG, -10.06 ± 0.20 ‰ and -6.05 ± 0.20 ‰ respectively; both fall within the expected range for NH_4^+ assimilation (-8 ‰ to –20 ‰). There was a significant difference in nitrogen isotopic composition between the cells grown on glutamine vs the cells grown on ammonium. Cells grown on glutamine were -3 ‰ (PyG/WT) to -9 ‰ (AcG/WT) more depleted in ^{15}N than when grown on ammonium. Removing the nitrogen from glutamine instead of having ammonium readily available had a clear impact on the $\delta^{15}N$ value for the cells. The impact of using nitrogen from glutamine instead of ammonium was much

greater for the cells grown with acetate as a carbon source (-9 ‰) than with pyruvate as a carbon source (-4 ‰). It is unclear why a change in carbon source would have this effect. It may be that for the cells to break down glutamine for nitrogen, pyruvate fermentation is needed for another source of electrons. When the cells are grown on acetate, they do not have this option available to them. The $\delta^{15}N$ values for the PyG or AcG media were somewhat confusing in that they did not appear to follow isotopic mass balance. Glutamine accounts for 99% of the nitrogen in the media, and so the media should be essentially isotopically identical to glutamine. This was not observed, as the media was 2 ‰ enriched in ^{15}N for PyG and 5 ‰ enriched for AcG. At first, I expected the difference in $\delta^{15}N$ values would also be the result of lyophilization; however, the nitrogen content measured in the media does not indicate that any nitrogen was lost. Regardless of whether the cell $\delta^{15}N$ values are compared to the media or to glutamine itself, they still fall within the typical range reported for N-assimilation. By comparing the cell $\delta^{15}N$ value to glutamine only, the per mil difference between the media and the two cell cultures becomes more pronounced. The cells grown on PyG have a $\Delta_{cell\text{-}glutamine}$ that is more than double that of cells grown on AcG (Table 4.1). When looking at the nitrogen content of the cells, growing on glutamine resulted in cells that were slightly richer in nitrogen compared to the cells that were grown with ammonium as the nitrogen source. Like the cells grown on ammonium as a nitrogen source, cells grown with pyruvate were slightly more nitrogen rich compared to cells grown with acetate.

Carbon content and isotopic values can also be compared between cells grown on PyG and AcG media, as well as the cells that were grown with ammonium as a nitrogen

source. The cells grown on PyG media were isotopically heavier (-27.12 ± 0.20 ‰) than the cells grown on AcG media (-27.68 ± 0.20 ‰). Although the range is only 2 ‰, the cells grown on PyG media were the isotopically lightest cells grown with pyruvate as the carbon source. These cells are also the only cells that are lighter than the pyruvate they grew on. Glutamine adds another carbon containing compound, so the cells may use the carbon from glutamine in a way that greatly fractionates the carbon from the glutamine source. Fractionating carbon from glutamine or needing to use pyruvate fermentation to extract nitrogen from glutamine are the likely causes of the fractionation difference seen in PyG/WT cells compared to PyA/WT or PyA/Y/WT cells. The $\delta^{13}C$ results for cells grown on AcG media are hard to interpret, largely due to the difference between the $\delta^{13}C$ of the two acetate compounds used. The AcG/WT cells had an $\delta^{13}C$ value that was lighter than the AcA/WT cells but heavier than the acetate-2 source. There were essentially two variables that were changed— the nitrogen source and bottle of acetate. Growing cells on acetate-2 as a carbon source and glutamine as a nitrogen source is a necessary step. The carbon content of the cells grown with glutamine mirrored the carbon content of the cells when grown on ammonium, as expected. The AcG/WT cells had a carbon content that was about average for my *H. modesticaldum* cells. Their carbon content was also higher than the PyG/WT cells.

The third nitrogen condition used to grow cells was the removal of nitrogen from the media to force the cells to fix nitrogen. Under this nitrogen condition, I expected the cells to have a significant difference in $\delta^{15}N$ compared to cells grown with the other two nitrogen sources. To fix nitrogen, *H. modesticaldum* is reported to have the molybdenum-

iron nitrogenase (Kimble and Madigan, 1992). This nitrogenase enzyme is known to result in only minor, if any, fractionation of nitrogen. Typically this fractionation range is only -3 to +1 ‰ (Fogel and Cifuentes, 1993; Hoering and Ford, 1960; Zhang et al., 2014), so I expect my cells grown in PyN to fall within this $\delta^{15}N$ range. I expected that by forcing the cells to fix nitrogen from the atmosphere, the nitrogen content of the cells would decrease. This is because the cells must now reduce the nitrogen prior to incorporating it into their biomass. Although nitrogen as an element isn't limiting in this case, it is more costly for the cell to use. Unlike the expectations for nitrogen content and composition, I did not expect the carbon content or composition to be affected by the change to nitrogen fixation. I expected the PyN/WT to be in the similar -25 to -28 ‰ $\delta^{13}C$ that was seen for PyG/WT and PyA/WT cells. For the cells grown on AcN media, I thought the $\delta^{13}C$ value would be similar to the AcG/WT cells because both media used acetate-2 as the carbon source.

The two growth conditions using N_2 as the sole nitrogen source can be compared to each other, as well as to the cells grown with glutamine and ammonium as nitrogen sources. The results for nitrogen fixing cells were the most unexpected. Although *H. modesticaldum* has the common nitrogenase, my cells do not fall into the typical $\delta^{15}N$ range for cells with this nitrogenase. Both PyN/WT and AcN/WT cells had more negative (depleted) $\delta^{15}N$ values of about -4 ‰. The genome of *H. modesticaldum* was checked for other nitrogenases that may have been missed, but none were found to be good matches by Dr. Redding. The $\delta^{15}N$ values seem to be more depleted than is typically reported for the molybdenum nitrogenase. In addition, both nitrogen fixing cultures have identical

$\delta^{15}N$ values ($p > 0.01$) and low standard deviations based on replicate measurements. This would seem to indicate *H. modesticaldum* do in fact fractionate nitrogen at -4 ‰ when fixing nitrogen. More research into the genome of heliobacteriaceae as a family and *H. modesticaldum* as a species needs to be done to determine the possibility that a different nitrogenase is being utilized. The minimal media I used contained vanadium. If the bacterium has a vanadium-iron nitrogenase, it could be in use. The vanadium-iron nitrogenase can fractionate from -3 to -6 ‰ (Zhang et al., 2014), meaning *H. modesticaldum* would fall into this range. However, at this stage the presence of an unusual nitrogenase is highly speculative. As expected, the nitrogen content of these cells was impacted because I forced the cells to fix nitrogen. In fact, the PyN/WT cells (12.20 ± 0.01 wt %) were only slightly affected and were near the average nitrogen content (12.40 wt %) for all the cells I evaluated. The AcN/WT cells deviated the most from this average; they had a nitrogen content of only 10.68 ± 0.07 wt %. The discrepancy in nitrogen content is likely due to the need for the cells to use pyruvate as an electron source to reduce the N_2. The PyN/WT cells can use some of their pyruvate for fermentation and gain a source of electrons in addition to a source of carbon. The AcN/WT cells do not have the ability to use pyruvate fermentation as a source of electrons and must use an alternative source of electrons. The media was limited to a single carbon source and no nitrogen besides N_2. With limited nutrients the cells do not grow as well, resulting in a very low nitrogen content relative to cells grown in the rest of the growth conditions.

With neither glutamine nor yeast present, the only carbon containing compounds present in the media were pyruvate or acetate. The $\delta^{13}C$ for the media matched the $\delta^{13}C$ values for their respective carbon source. Like the nitrogen isotopic data, the carbon isotopic data for nitrogen fixing cells was very unexpected. The PyN/WT cells were much heavier than the pyruvate source, with the $\Delta_{cell\text{-}pyruvate}$ at +3.60 ‰. If pyruvate fermentation is the source of electrons for nitrogen fixation, it may be impacting the carbon isotopic fractionation as well. When the cells are fermenting pyruvate, light CO_2 may be the biproduct of the reaction. If the cells are losing light CO_2, then the remaining carbon would be heavier. The loss of light carbon and retention of heavy carbon would explain why the nitrogen fixing cells that grew on pyruvate are the most ^{13}C rich. The AcN/WT cells were the lightest of any cell culture at -32.34 ± 0.30 ‰. This value is nearly identical to the media and acetate-2 component $\delta^{13}C$ values. Unlike the AcG/WT and AcA/WT cells, there is no fractionation of acetate-1 or aceate-2, either positively or negatively. It is unclear why fixing nitrogen vs assimilating nitrogen would impact the cells grown acetate as the pyruvate fermentation would not be occurring in either culture. Similar to the nitrogen content, the carbon content of PyN/WT cells were just slightly below average (42.91 wt %), while the carbon content of AcN/WT cells was significantly lower at 39.63 wt %. Not only was this the only cell culture below 42 wt %, but it was also below 40 wt % indicating these cells had a very hard time growing.

4.3 Citrate Synthase Knockout

In addition to changing carbon and nitrogen sources, I was able to examine differences between wild type cells and genetically altered mutant cells. The mutant cells

were missing the citrate synthase (CS) enzyme, and I refer to this cell type as the citrate synthase knockout (CSKO). By eliminating the CS enzyme, the reductive TCA cycle (rTCA) becomes a linear pathway from acetate to citrate (Fig 4.1). Acetyl-CoA is no longer able to be used to create citrate directly and preform the TCA cycle in the forward direction. The cells are known to grow on glutamine, so the mutant cells were grown on the PyG and AcG media to avoid any unwanted volatilization during lyophilization. It is currently unknown if the mutant cells would grow in PyN and AcN media. I did not change the nitrogen source, so I expected the PyG/CSKO cells to have similar $\delta^{15}N$ value to the PyG/WT cells. I also anticipated the same for the mutant cells grown on the AcG media. The nitrogen content of the cells should not be highly impacted by this knockout, as the cells are grown with glutamine as a readily available source of reduced nitrogen. I expected the carbon content of the two mutant cell cultures to be lower than the carbon content of the wild type cells. By removing access to the oxidative TCA cycle (oTCA), I projected cell growth to be hindered. For *E. coli*, it is known that cells grow exponentially worse with limited CS enzyme activity (Wu et al., 2020). Another known impact of knocking out the CS gene is an influence on the flux of oxaloacetate in equilibrium with malate. Malate is not favored in the reaction, reducing not only this reaction, but the entire rTCA cycle. Any changes to the carbon content or isotopic composition are likely centered around changes to this reaction and the reduced abundance of the subsequent compounds that are no longer able to be made in the forward direction. I was unable to find literature on the effect of knocking out citrate synthase on the $\delta^{13}C$ value of cells.

The media used to grow the mutant cells was the same media that was used to grow the wild type cells on glutamine (PyG and AcG). As mentioned previously, the $\delta^{15}N$ value of both media were not what I expected them to be with glutamine as the only nitrogen source in the media. The mutant cells follow the same pattern as the wild type cells with cells grown in PyG, as they were isotopically lighter than cells grown in AcG. Notably, for both carbon sources, the mutant cells are about 1.5 ‰ heavier than their wild type counterparts. The change in metabolism caused by the remove of the CS enzyme appears to be independent of whether pyruvate or acetate is used as the carbon source. Similar to the wild type cells, the fractionation of cells grown in pyruvate compared to glutamine is about double that of cells grown in acetate (Table 4.1). The nitrogen content of the mutants were higher for both growth conditions, with PyG/CSKO cells being higher than the average. The PyG/CSKO had the highest carbon content of any cells grown, except for PyA/YD/WT cells.

The carbon content of the mutant cells followed a similar trend as the nitrogen content. The mutant cells were richer in carbon than the wild type for both growth conditions. In contrast to the nitrogen content values, mutant cells grown on both pyruvate and acetate had carbon contents higher than the average for the cells I measured. In fact, they had two of the highest carbon content values for all cells I investigated. It is possible that by not being able to complete the rTCA cycle, the cells are forced to store more carbon-rich compounds instead of repurposing them when they are able to complete the cycle. The knockout of the CS enzyme did not seem to have much of an impact on the $\delta^{13}C$ of the cells grown on pyruvate. The PyG/CSKO cells were less than 1 ‰ heavier

than the PyG/WT cells. There was a greater effect on the carbon isotopic composition of the AcG/CSKO cells. The AcG/CSKO cells were heavier than the AcG/WT cells by almost 3 ‰. This created an even greater difference between mutant and wild type $\delta^{13}C$ values. The mutant cells grown on acetate had a higher $\delta^{13}C$ value than the mutant cells grown on pyruvate. By eliminating the CS enzyme, acetyl CoA cannot be converted to citrate. If wild type *H. modesticaldum* does not use pyruvate fermentation when exposed to light and a reduced nitrogen source, such as with my PyG/WT cells, the reaction from pyruvate to acetyl CoA and acetyl CoA to citrate may not be used as much. Starting with acetate forces the cells to create acetyl CoA regardless; hence, the CS enzyme may be more active when growing on acetate. This would result in a bigger change of $\delta^{13}C$ for cells grown on acetate than pyruvate when the CS enzyme is knocked out.

Table 4.1. Fractionation of carbon isotopes from cells to media or their carbon source. $\Delta_{1\text{-}2}$ values are calculated by $\delta^{15}N_1$ - $\delta^{15}N_2$. Values are expressed in ‰. Error is propagated from the error of each $\delta^{15}N$ value.

Cells	$\Delta_{cell\text{-}media}$ (‰)	$\Delta_{cell\text{-}ammonium}$ (‰)	$\Delta_{cell\text{-}glutamine}$ (‰)	$\Delta_{cell\text{-}N_2}$ (‰)
PyA/Y/WT	-7.66 ± 0.30	-5.95 + 0.28		
PyA/YD/WT	-6.06 ± 0.65	-4.35 ± 0.64		
AcA/Y/WT	-10.04 ± 0.28	-4.11 ± 0.28		
PyA/WT	-11.97 ± 0.28	-12.08 ± 0.28		
AcA/WT	-49.78 ± 1.70	-13.77 ± 0.28		
PyG WT	-9.74 ± 0.68		-7.73 ± 0.28	
AcG WT	-8.46 ± 0.94		-3.72 ± 0.28	
PyG CSKO	-8.59 ± 0.68		-6.58 ± 0.28	
AcG CSKO	-7.00 ± 0.94		-2.26 ± 0.28	
PyN/WT	-0.47 ± 3.60			-3.96 ± 0.20
AcN/WT	-4.02 ± 1.99			-4.04 ± 0.20

Table 4.2. Fractionation of carbon isotopes from cells to media or their carbon source. $\Delta_{1\text{-}2}$ values are calculated by $\delta^{13}C_1$ - $\delta^{13}C_2$. Values are expressed in ‰. Error is propagated from the error of each $\delta^{13}C$ value.

Cells	$\Delta_{cell\text{-}media}$ (‰)	$\Delta_{cell\text{-}pyruvate}$ (‰)	$\Delta_{cell\text{-}acetate}$ (‰)
PyA/Y/WT	-0.67 ± 0.28	0.28 ± 0.28	
PyA/YD/WT	-0.73 ± 0.31	0.22 ± 0.31	
AcA/Y/WT	-0.95 ± 0.34		-8.92 ± 0.28
PyA/WT	0.36 ± 0.28	0.34 ± 0.28	
AcA/WT	-4.29 ± 0.36		-4.6 ± 0.28
PyG WT	-7.04 ± 0.28	-1.3 ± 0.28	
AcG WT	-8.66 ± 0.83		4.17 ± 0.28
PyG CSKO	-6.61 ± 0.28	-0.87 ± 0.28	
AcG CSKO	-6.16 ± 0.83		6.67 ± 0.28
PyN/WT	3.49 ± 0.28	3.6 ± 0.28	
AcN/WT	-0.12 ± 0.36		-0.49 ± 0.36

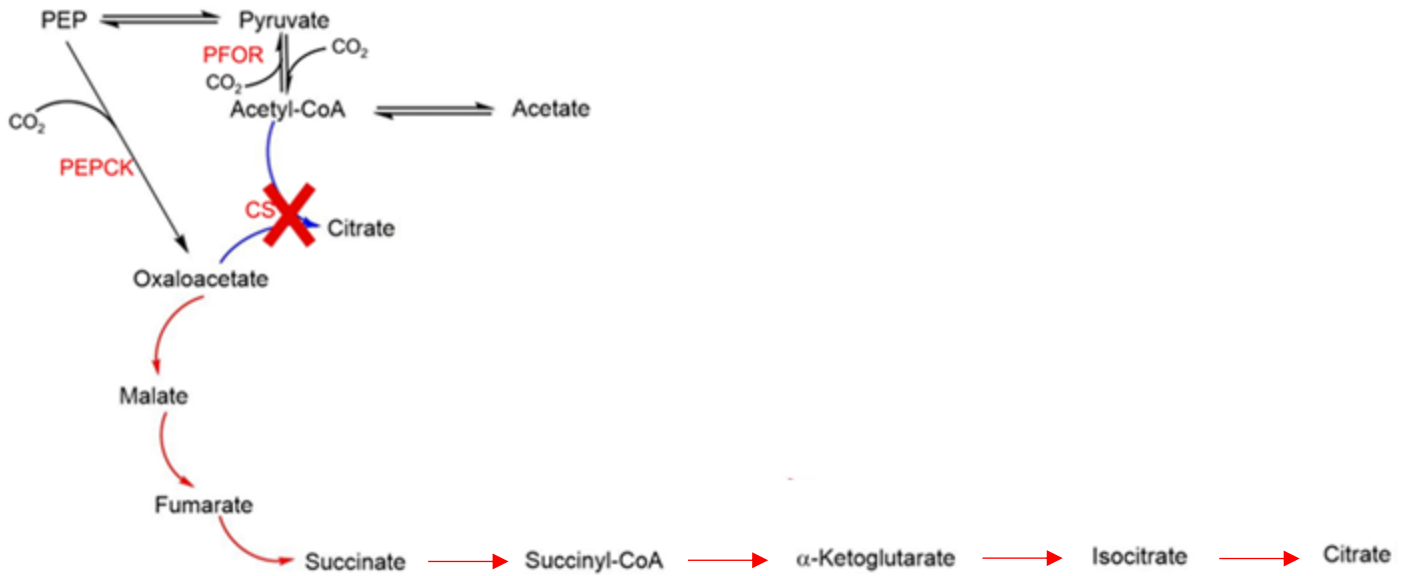

Figure 4.1 An rTCA cycle that is missing the citrate synthase enzyme. This causes the cycle to become linearized. Modified from figure 1.2.

CHAPTER 5
SUMMARY AND CONCLUSIONS

Heliomicrobium modesticaldum is a unique bacterium in that it can use light as a source of energy but cannot perform photosynthesis. By not being able to perform CO_2 fixation, it relies on organic carbon as its source of carbon rather than carbon dioxide like typical photosynthesizers. *H. modesticaldum* also uses organic molecules as a reductant source, thus making it a photoorganoheterotroph under the best conditions. Under suboptimal conditions in the dark, *H. modesticaldum* can also grow as a chemoorganoheterotroph by getting energy from chemical reactions as opposed to light. *H. modesticaldum* also has the ability to fix nitrogen from atmospheric N_2 if the environment lacks reduced nitrogen. Here we changed the environmental conditions of *H. modesticaldum* cultures, inducing changes in the biochemistry of the cells. This allows me to measure the impact to the carbon and nitrogen content of the cells, as well as the carbon and nitrogen isotopic signatures.

The cells were first grown using known optimal growth conditions, which included media with yeast to provide a preferred carbon and nitrogen source. Although the cells did grow well, the yeast caused issues with the interpretation of the isotopic signature of the cells. Yeast has an unknown composition, so claiming to know the exact processes used by the cells or any difference compared to other cell cultures is unreasonable. For this reason, interpreting any differences between cells grown with

yeast in the light as photoorganoheterotrophs vs cells grown with yeast in the dark as chemoorganoheterotroph must be done with caution. Unfortunately, cells did not grow in the dark when yeast was not present.

Changing the nitrogen source provided to the cells allowed me to examine the effect of nitrogen fixation vs nitrogen assimilation on the content and isotopic signature of carbon and nitrogen in the cells. *H. modesticaldum* had typical carbon and nitrogen contents as well as typical carbon and nitrogen isotopic signatures for cells assimilating nitrogen. The cells that were forced to fix nitrogen were unique in that the carbon and nitrogen content of these cells were very low. The low content values were especially low for the nitrogen fixing cells grown on acetate. Without access to pyruvate fermentation, fixing nitrogen became a limiting factor, as there was no abundant reductant source. Forcing nitrogen fixation also had a noticeable impact on the isotopic signatures of the cells. The nitrogen fixing cells grown on acetate had $\delta^{13}C$ values that were isotopically light compared to the nitrogen assimilation cells. In contrast, the nitrogen fixing cells grown on pyruvate had $\delta^{13}C$ values that were isotopically heavy compared to the nitrogen assimilation cells. The $\delta^{15}N$ values were lighter than expected at -4 ± 0.2 ‰ rather than the expected range of -3 to +1 ‰. Although the $\delta^{15}N$ values were lighter, they were the same regardless of the carbon source used to grow the cells. The $\delta^{15}N$ were outside the expected range for molybdenum-iron nitrogenase based nitrogen fixation for cells. Further study is needed to investigate the mechanism for the differences in $\delta^{15}N$ between *H. modesticaldum* and organisms that use the molybdenum-iron nitrogenase. The

nitrogenase catalyst active in our *H. modesticaldum* cultures may be novel or may contain a less common cofactor. This offers an exciting prospect for future research.

A novel strain of *H. modesticaldum* was modified to remove its citrate synthase enzyme. The carbon and nitrogen content of the mutant cells was higher than the wild type cells, which was unexpected. The mutant cells had $\delta^{15}N$ values that were slightly heavier than the wild type cells but followed the previously observed pattern of cells grown with pyruvate having isotopically lighter nitrogen compared to cells grown with acetate. The mutant cells also had heavier $\delta^{13}C$ values compared to the wild type cells. The pattern between acetate and pyruvate was not the same for $\delta^{13}C$, as the cells grown on acetate were heavier than cells grown on pyruvate for the mutant cells but not for the wild type cells. The shift of the $\delta^{13}C$ and $\delta^{15}N$ values for the mutant cells indicate that removing citrate synthase has a measurable impact on the biochemistry of the cell. Further evidence for this is illustrated by the AcG/CSKO cells that became almost 3 ‰ heavier than AcG/WT cells. By removing the citrate synthase enzyme, the cells are forced to use a linearized version of the rTCA cycle. The removal of citrate synthase also means acetyl CoA cannot be converted to citrate directly. The removal of the ability to directly make citrate from acetyl CoA seems to have impacted the cells grown on acetate more than the cells grown on pyruvate. Consequently, the carbon source has an impact on what reaction the cells are performing to grow.

In general, *H. modesticaldum* did not reflect the isotopic composition I expected. *E. coli* had a carbon isotopic composition that reflected the carbon source it was using to grow. For example, *E. coli* had a $\delta^{13}C$ of -9.6 ‰ when grown on glucose that had a $\delta^{13}C$

value of -9 ‰ (Blair et al., 1985). Wild type *H. modesticaldum* is also a heterotroph, but did not look like the carbon sources it grew on. There is no isotopic data for cells grown on glutamine, and so ammonium assimilation was the closest comparison. *Vibrio harveyi* (-15 ‰ $\delta^{15}N$) and *Marichromatium purpuratum* (-7 ‰ $\delta^{15}N$) are two bacteria that use ammonium assimilation to incorporate nitrogen. Except for mutant *H. modesticaldum,* my cells had $\delta^{15}N$ that were similar to these known values. *Rhodopseudomonas palustris* is an example of a bacteria that fixes nitrogen using the molybdenum-iron nitrogenase and has a $\delta^{15}N$ value of about -2 ‰. No other individually measured bacteria that use the molybdenum-iron nitrogenase have a $\delta^{15}N$ value less than -2 ‰. The wildtype H. modesticaldum that fixed nitrogen did have a $\delta^{15}N$ value that was less than -2 ‰ at -4 ‰. Isotopic data for individual organisms is relatively limited compared to the total number of organisms studied. *H. modesticaldum* specifically is also part of a lineage that is limited in the isotopic data available. Having limited data on close relatives of *H. modesticaldum* is problematic as comparing my results to the general patterns for all bacteria is not sufficient in determining the isotopic uniqueness of this organism. Other firmicute nitrogen fixers may fall outside of the general range seen in the rest of the bacterial world. Problems from comparing an individual organism to the general trends as opposed to individual closely related organisms will continue until more isotopic values for individual organisms are measured.

Further experiments need to examine the nitrogenase enzyme(s) available to *H. modesticaldum* to determine the atypical isotopic signature for the expected nitrogenase of *H. modesticaldum*. Other mutant *H. modesticaldum* can be grown and compared to my

preliminary results to determine the effect of future changes on isotopic signature. Relationships between isotopic signature and metabolic pathways can be studied as more growth conditions are used and future mutant *H. modesticaldum* are grown. This will give us a baseline for understanding potential metabolic pathways and their biosignatures for ancient Earth organisms or possible organisms on other planets.

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