

**Development and application of liquid
chromatography-tandem mass spectrometry
methods to the understanding of metabolism and
cell-cell signaling in several biological systems**

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ABSTRACT

Liquid chromatography tandem mass spectrometry has become a powerful tool for investigating biological systems. Herein we describe the development of both isotope dilution mass spectrometry methods and targeted metabolomics methods for the study of metabolic and cell-cell signaling applications.

A putative yeast enzyme was characterized by discovery metabolite profiling, kinetic flux profiling, transcriptomics and structural biology. These experiments demonstrated that the enzyme shb17 was a sedoheptulose bisphosphatase that provides a thermodynamically dedicated step towards riboneogenesis, leading to the redefinition of the canonical pentose phosphate pathway.

An extension of metabolic profiling and kinetic flux profiling methods was developed for a set of symbiotic marine microorganisms. Carbon flux from the most abundant photosynthetic organism, *Prochlorococcus*, to a symbiotic *Alteromonas* was observed in liquid coculture. These methods enable a more biologically relevant assay for marine species and will lead to a better understanding of carbon flux in the oceans.

Energy taxis refers to the active migration of bacteria in response to electron transport system related signals. The second messenger cyclic-di-GMP provides a link between the metabolic signals and motility. Quantitation of c-di-GMP helped characterize the nature of this regulation.

Autoinducer-2 is a small sugar produced by a large variety of bacteria that is proposed to be a universal quorum sensing signal. The quorum sensing function of autoinducer-2 is disputed because it is produced by an enzyme of the activated methyl cycle, leading to an alternate hypothesis that it is simply a metabolic byproduct. Herein a method for the detection of autoinducer-2 is developed to enable studies of its signaling role and biosynthetic regulation. These studies demonstrated that autoinducer-2 does not function as a signal in all species. Further, metabolic experiments indicated that the metabolic impact of LuxS dysfunction was small and could be mitigated by recycling oxidized glutathione. Together, these data indicate that neither hypothesis is adequate. Evidence is provided that autoinducer-2 suppresses the immune system, by the interruption of cytokine signaling, implying that autoinducer play a protective role during host colonization.

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List of Abbreviations

[]	concentration
ABPP	activity based protein profiling
AHL	acylhomoserine lactone
AI	autoinducer or quorum sensing signal
AI-1	autoinducer-1
AI-2	autoinducer-2
AIP	peptide autoinducer
AMC	activated methyl cycle
<i>A. naeslundii</i>	<i>Actinomyces naeslundii</i>
ATP	adenosine-triphosphate
CE	capillary electrophoresis
DHAP	dihydroxyacetone phosphate
DPD	4,5-dihydroxy-2,3-pentanedione
E4P	erythrose-4-phosphate
<i>E. coli</i>	<i>Escherichia coli</i>
FBP	fructose-1,6-bisphosphate
GAP	glyceraldehyde-3-phosphate
GC	gas chromatography
HAI-1	harveyi autoinducer-1
HILIC	hydrophilic interaction chromatography
HPLC	high performance liquid chromatography
KFP	kinetic flux profiling
LC	liquid chromatography
LOD	limit of detection
MRM	multiple reaction monitoring
MS	mass spectrometry
NADH	nicotinamide adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide phosphate
NMR	nuclear magnetic resonance
O1P	octulose-1-phosphate
O8P	octulose-8-phosphate
OBP	octulose-1,8-bisphosphate
PEEK	polyether ether ketone
PPP	pentose phosphate pathway
Q	quadrupole
QS	quorum sensing
R5P	ribose-5-phosphate
RP	reverse phase
S/N	signal to noise ratio
S1P	sedoheptulose-1-phosphate
S7P	sedoheptulose-7-phosphate
SAH	S-adenosylhomocysteine
SAM	S-adenosylmethionine
SBP	sedoheptulose-1,7-bisphosphate
<i>S. cerevisiae</i>	<i>Saccharomyces cerevisiae</i>
<i>S. oralis</i>	<i>Streptococcus oralis</i>

<i>S. typhimurium</i>	<i>Salmonella typhimurium</i>
SCX	strong cation exchange
<i>shb17</i>	sedoheptulose-1,7-bisphosphatase
SRH	S-ribosylhomocysteine
SRM	single reaction monitoring
TOF	time-of –flight
UPLC	ultra-high performance chromatography
<i>V. harveyi</i>	<i>Vibrio harveyi</i>
Xu5P	xylulose-5-phosphate

CHAPTER I

METABOLOMICS AND SYSTEMS BIOLOGY

The term systems biology describes the field of science that studies perturbations of an intact biological system by using global profiling techniques to establish the relationship between the elements of the system and build mathematical models to explain the emergence of new phenotypes.^{1,2} This approach can be contrasted with the more traditional reductionist approach, which studies individual elements in detail. While reduction has been extremely useful in identifying system elements and determining some interactions, it cannot be easily extrapolated to larger systems due to the complexity and robustness of biological networks. Several global profiling techniques are used in systems biology, each with its own advantages and limitations.

1.1 Genomics

Genomics is the study of the genome of an organism, and often entails sequencing the entire genetic makeup of the organism. This information provides a blue print describing the capabilities, or genes that could be transcribed, of that organism. Due to the genetic variation between individuals, the genome also reveals markers that allow identification of the donor species and individual. By extension, meta-genomics looks at all of the genomes in a larger system, ie. ocean water, to study the number and types of organisms living in that system.³ Genomics studies however are limited to information about the capabilities of the system, but not the current state of a system. It is therefore not a useful method for studies looking at a short-term cellular response to a perturbation; however the data from genomics studies informs data interpretation for these experiments.

Genomics studies rely on one of several sequencing technologies, many of which were developed concurrently with the Human Genome Project. The Human Genome Project was an international collaborative effort mostly completed in 2003, which ventured to sequence the entire human genome. The project provided the impetus for the development of next-generation sequencing technologies and produced a substantial data set that enabled the extension into other types of global profiling. Current popular gene sequencing technologies include the short read platforms Illumina/Solexa and ABI SOLiD which until recently have require a reference genome for

assembly, and the long read platform Roche 454 for sequencing genomes with no available reference genome.⁴

1.2 Transcriptomics and Proteomics

Transcriptomics and proteomics were the next systems biology tools to develop. These tools are an improvement on genomics because mRNA and protein concentrations report the result of some regulatory stages including sigma factors, transcription factors tied to signal transduction and other regulators of gene transcription and translation. In essence these technologies report what the biological system is attempting to do.

The workhorse technology for transcriptomics is the microarray. Microarrays consist of single stranded DNA printed or synthesized on a surface (typically glass) referred to as a chip. Fluorescently labeled cDNA made from isolated mRNA can be incubated with the chip which allows hybridization to occur. After unhybridized cDNA is washed away, lasers can “read” the different fluorescent labels to quantify how much cDNA hybridized to each probe and from which sample.⁵ A search for microarrays in Web of Science turns up a wide variety of applications (>10,000 hits) including prediction of breast cancer outcomes, detection and characterization of respiratory viruses, and understanding kidney development. One problem with this technology is that mRNA is not always translated into protein; and if it is, the mRNA may be translated once or several times. Thus transcription levels do not necessarily reflect the amount of each protein in the cell, and this limitation was the impetus for the development of proteomics.

Proteomics experiments rely primarily on nanospray - tandem mass spectrometry for the identification and sequencing of peptides resulting from digestion of proteins. Peptides undergo fragmentation by breaking at the amide bond which allows peptide sequencing in MSⁿ experiments. However, de novo sequencing is cumbersome and some amino acids (leucine and isoleucine) share the same mass-to-charge ratio (m/z) making a full sequence impossible. In most cases peptide m/z measurements in conjunction with the m/z data of several peptide fragments can be attributed to a protein based on the predicted amino acid sequence from a reference genome in an automated fashion. While quantitative experiments comparing two treatment groups can be conducted this way, there are many opportunities for the introduction of error. Labeling

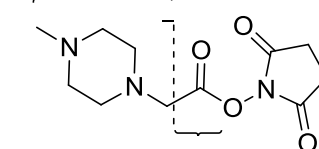
Total mass = 145				
Reporter Group		Reporter Group	Heavy Isotopes in Reporter Group	Mass Balance Group
		<i>m/z</i>	<i>m/z</i>	<i>m/z</i>
		114	^{13}C	31
		115	$^{13}\text{C}_2$	30
		116	$^{13}\text{C}_2\ ^{15}\text{N}$	29
		117	$^{13}\text{C}_2\ ^{15}\text{N}$	28
Mass Balance Group				Heavy Isotopes in Mass Balance Group
				$^{13}\text{C}\ ^{18}\text{O}$
				^{18}O
				^{13}C

Figure 1.1. Structure of iTRAQ reagents.

reagents have been developed to enable relative quantitation between samples.⁶ For example iTRAQ reagents may be reacted with as many as four different samples. The reagent undergoes reaction with any free amines in the sample peptide mixture, creating a new amide bond. The samples can then be mixed and relative peptide concentrations measured by MS/MS experiment. Because the iTRAQ reagents are isobaric, the parent *m/z* of a peptide will be identical for all samples. Upon fragmentation however, product peaks unique to each sample can be measured. The ratio of these product ion signals describes the relative abundance of the peptide in the samples.^{7,8} Naturally occurring post-translational modifications are often less stable than this chemistry, and a current challenge in this field is the preservation of these modifications during extraction and their inclusion in automated sequencing techniques.⁹ This would be a particularly strong advancement for the field since proteomics research could then be able to address questions of protein regulation.

1.3 Functional Proteomics

Posttranslational modification is a very important part of what determines an enzyme's catalytic activity. Beyond incorporation of necessary cofactors, cleavage of proenzymes, phosphorylation and ubiquitylation, to name a few, all regulate the active state of enzymes.¹⁰ The global protein profiling discussed to this point has primarily focused on counting or comparing the number of a particular protein present. Alternatively, the total catalytic activity or amount of active protein could be quantified, and this is the premise of activity based protein profiling (ABPP). The large family of serine hydrolases was the first characterized by ABPP using a probe containing a reactive group designed to covalently bind with the active site and an experimental

handle containing a fluorescent group or biotin.¹¹ These handles allow separation, visualization, and quantitation via western-blot analyses. The biotin probes can also be used for purification of active proteins which can then be digested and identified using proteomics mass spectrometry techniques.¹² Such methods have enabled both drug discovery and off-target drug interaction screening¹³ as well as identification of new disease markers.¹⁴ Side-by-side comparisons of ABPP and cDNA microarrays of breast tissue have shown that ABPP is better at classifying tumor types, demonstrating that methods accounting for all types of activity regulation are more efficient than those methods that account for only some of them.¹²

1.4 Intro to Metabolomics

Like activity based protein profiling, metabolomics captures the current metabolic state of the cell, except in this case the small molecule concentrations and turnover rates are measured. The metabolome is made up of about ~ 700 conserved small polar metabolites,¹⁵ while there are upwards of 30,000 biologically active lipids in the Lipid Maps Structure Database;¹⁶ and in more prolific species such as the plant kingdom there may be >10,000¹⁷ polar metabolites. Although much of metabolism is conserved, a recent study has demonstrated how chemically different three sample types from the same patient can be. The study characterized metabolites in plasma, urine, and prostate tissue and showed that of 1265 metabolite signals measured, 46% were unique to one of the three sample types. It is important to note that the study limited further analysis to the 626 signals present in tissue samples, and identified the metabolite responsible for 175 of these.¹⁸

The metabolome presents unique technical challenges to the development of global measurement methods, when compared to the development of other systems biology techniques. Metabolism can change on the order of seconds, making it necessary to work quickly as changes due to the stress of the isolation and extraction process can appear in the data.¹⁹⁻²¹ The chemical properties of these molecules are also much more varied, and there is not a repeating chemical handle for analysis similar to, for example, the amide bond in proteomics experiments. This variety poses problems at all experimental stages. For example, hydrophilic and hydrophobic metabolites partition differently during extraction, molecules with low pK_a s ionize under different conditions

than those with high pK_a s, and dissimilar fractionation behavior between molecules limits the capabilities of automated data analysis software.

There are two approaches to metabolomics experiments. The first is an untargeted approach where the goal is to measure the largest number of metabolites and identify those metabolites that change. These experiments typically use a high-resolution mass spectrometer (MS) in order to predict the molecular formula of the metabolite or NMR to identify structural characteristics. These techniques are often inadequate for identification and require further experiments to validate the identity of changed metabolites. Therefore, they primarily provide a metabolic “fingerprint” and launch further more in-depth studies. Among this type of experiment is a technique called discovery metabolite profiling.²² This method can identify a previously unknown metabolite and simultaneously assign an enzyme that uses it as a substrate. Alternatively, an unknown gene product can be assigned a function based on the identification of its substrate. This is accomplished by deleting a specific gene from an organism (or screening a library of strains with mutations in individual genes) and then looking for an increase in the concentration of its substrate and a drop in the concentration of its products (Figure 1.1). While homeostasis is preserved in many cases due to multiple regulatory components in metabolic pathways,²³ this change in concentration has been observed in several studies,^{22,24} two of which are reported herein.

The second approach is a targeted approach, where the experiment quantifies a set of previously characterized metabolites included in a library. These methods utilize a mass analyzer capable of quantitation such as a quadrupole and/or time-of-flight, and because results are being compared to a library, high resolution MS is not as necessary.

Typically metabolomics experiments have been conducted on two types of instrumentation NMR and mass spectrometry. NMR experiments are excellent discovery platforms because of the innate structural information gathered for each unknown. However the sensitivity is not as good as a mass spectrometer and for this reason is used less often for quantitative experiments. Our methods use a targeted MS approach and thus further details on the experimental setup follows.

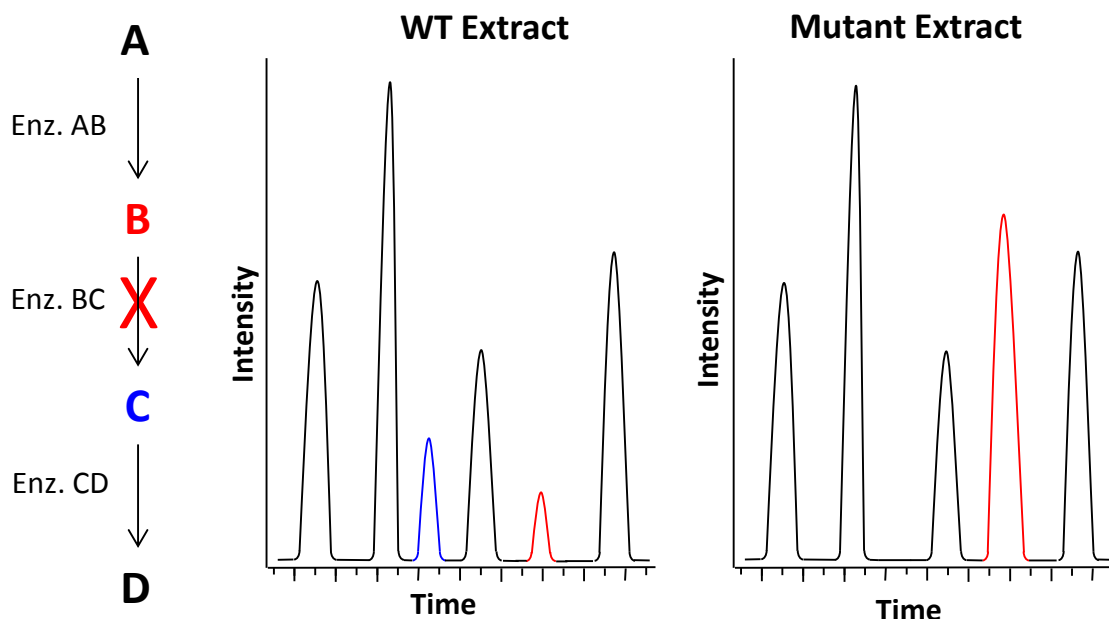


Figure 1.2 A discovery metabolite profiling experiment characterizing hypothetical enzyme BC. The substrate B (red) accumulates in the mass spectrum of an EnzBC⁻ mutant, and the product C (blue) signal disappears.

1.5 Sample Preparation and Extraction

As mentioned previously, metabolism can change in seconds, making long isolation steps such as centrifugation problematic. Experimental results can be obscured by the measurement of cold shock, sheer stress, and other results of handling during the extraction process. Hence, sample preparation is a very important consideration in experimental design.²⁰ Because biological systems vary in chemical and physical properties, isolation and extraction techniques often vary by sample type. Extraction methods may also be optimized for a certain subset of metabolites depending on the objective of the experiment. A 2007 article reported a side-by-side comparison of several common extraction methods and solvents in *Escherichia coli*.¹⁹ The authors concluded that several of the methods typically used allowed degradation of sensitive metabolites. An acidic 2:2:1 acetonitrile:methanol:water extraction solvent best preserved the metabolites sensitive to degradation while not sacrificing the efficient extraction of a breadth of metabolites. Metabolites from bacteria isolated by centrifugation and filtration were also compared. These experiments concluded that centrifugation allows too much

time for the bacteria react to their changing environment, while the filtration method greatly speeds the process and prevents many of these changes.²⁰ We have adapted these methods for our experiments analyzing bacteria and yeast. Suggested extraction methods for attached cells are similar, however media can be aspirated directly from the cells and then cold solvent added immediately to the cells.²⁵

As the pharmaceutical, biotechnology and medical industries begin to adapt these technologies, other considerations for clinical utility and current clinical sampling procedures must be made. For example, chemical treatment of serum with anticoagulants or tissue specimens with formaldehyde causes changes in the chemical make-up of the sample, and steps must be taken to account for these in the analytical design.²⁶

1.7 Common Separation Techniques

Usually MS metabolomics involves some type of chromatography technique. The most common are liquid chromatography (LC) and its high pressure cousins, gas chromatography (GC), and capillary electrophoresis (CE). Each has pros and cons. The earliest metabolomics methods used gas chromatography. Gas chromatography techniques can only separate compounds that are highly volatile and thus they are good for analyzing lipids or derivatized metabolites, but not high boiling polar and charged metabolites. Derivatization can be a powerful technique capable of enhancing the sensitivity and selectivity of an assay, but there are drawbacks to using it in complex, chemically undefined samples. There is always concern that the reactions do not continue to completion, and if this is the case, differences in chemical reactivity can cause one component to react over another and bias quantitation. GC methods built the foundation of lipidomics experiments, but have largely been supplanted by current LC methods.^{27,28}

Capillary electrophoresis methods separate molecules by overall charge and size, thus it is a good method for separating ionic or highly charged metabolites. Non-polar metabolites can be separated by CE as well with the addition of additives such as the surfactant SDS. This method produces very narrow peaks, allowing shorter run times, and therefore CE is most commonly interfaced with an MS capable of high acquisition rates such as time of flight (TOF). Drawbacks to this method include possible

incompatibility between additives and MS, limiting the scope of metabolites analyzed. The technique can also introduce metabolite degradation products due to electrochemical reactions during separation and has poor reproducibility and low sensitivity when compared to GC and LC methods.^{17,29}

Liquid chromatography has many advantages over the two methods mentioned previously for the analysis of core carbon and nitrogen metabolism. There are many types of stationary phases available for optimizing separation without fundamentally changing instrumentation. Reverse-phase chromatography is not ideal for metabolomics because the polar metabolites are not well retained on the column.^{30,31} This problem can be corrected by the addition of ion pairing agents.³² Hydrophilic interaction chromatography (HILIC) offers another appealing solution since it can retain polar metabolites and uses a sample solution and MS compatible solvent system. The separation theoretically does not need to be different for positively and negatively ionizing metabolites, although in practice two chromatography modes seems to offer the best separation.³⁰ A discussion of a two-dimensional LC separation is described in Chapter 2. The advent of ultra-high performance chromatography (UPLC) brought better reproducibility, increased signal to noise ratios, lower ions suppression and shorter analysis times. UPLC can also detect more metabolites than similar HPLC methods.³³ After separation, the eluent can be sent directly to the MS detector via an electrospray ionization source.

1.8 Mass Spectrometry Methods

In order to prevent further spectral complexity due to multiple peaks per molecule analyzed and allow meaningful tandem MS methods, a soft ionization method is preferred for metabolomics experiments. The ionization in GC-MS is an exception as it typically uses electron impact and chemical ionization techniques. The most common ionization source for CE and LC separated metabolites is electrospray ionization (ESI), however nanospray, atmospheric pressure chemical ionization (APCI), and atmospheric pressure photoionization (APPI) approaches have also been utilized (Figure 1.2).³¹ ESI uses a high voltage across a metal capillary to create a charged conical spray. As the solvent in each of the fine droplets evaporates the charge is left on the solute molecules.³⁴ APCI is somewhat more likely to cause molecular fragmentation in source.

Droplets of eluent are dried by a heat source to a fine mist, which passes by a corona discharge needle. The needle primarily ionizes the solvent and nebulizing gas, which then participate in proton transfer reactions with the molecules of interest.³⁵ Molecules enter the gas phase in APPI similarly to APCI, but a UV lamp is responsible for ionization. Photons can either cause a molecule to lose an electron, forming a radical cation, or be absorbed by a molecule which then is transferred to a high energy state. At atmospheric pressure molecules in the source continue to react and create a spectrum of ions. An easily ionized dopant is added to increase the ionization efficiency.³⁶ Other ionization strategies, including matrix-assisted laser desorption ionization (MALDI) matrix-suppressed laser desorption ionization (MSLDI), desorption ionization on porous silicon (DIOS), and desorption electrospray ionization (DESI) have also been successful.³¹

There are two technical issues necessary to recognize at this stage. First, changes in ionization efficiency may occur between samples and lead to systematic experimental error. Typically this occurs in our ESI instrumentation by partial occlusion of the ion transfer tube due to a buildup of salts and other contaminants on the surface. This can be corrected for by randomizing sample order and by inclusion of an internal standard. The second technical issue is called ion suppression, which occurs when more than one compound coelutes from the column and the ionization of low abundance or weakly ionizing species is suppressed by the competitive ionization of other specie(s).³⁷ This phenomenon often results in a split chromatographic peak (Figure 1.2 panel D), poor detection, and limited quantification of the weakly ionizing species. The utility of short chromatographic run times is limited in ESI-MS by ion suppression related interference with quantitation.

Several mass analyzers have proven useful for metabolomics techniques including time of flight (TOF), quadrupole (Q), Fourier transform (FTMS), and both ion and orbitraps (Figure 1.3).³¹ Quadrupoles separate molecules by their movement through an oscillating electric field created between the quadrupole rods. These instruments are compact, stable, robust, and inexpensive making them a very popular choice for either stand-alone instruments or coupled to other analyzers for tandem MS purposes. Triple quadrupole (QqQ) instruments may be run in multiple reaction monitoring mode (MRM), where the instrument scans through a programed list of reactions at a rate of ~50 ms per

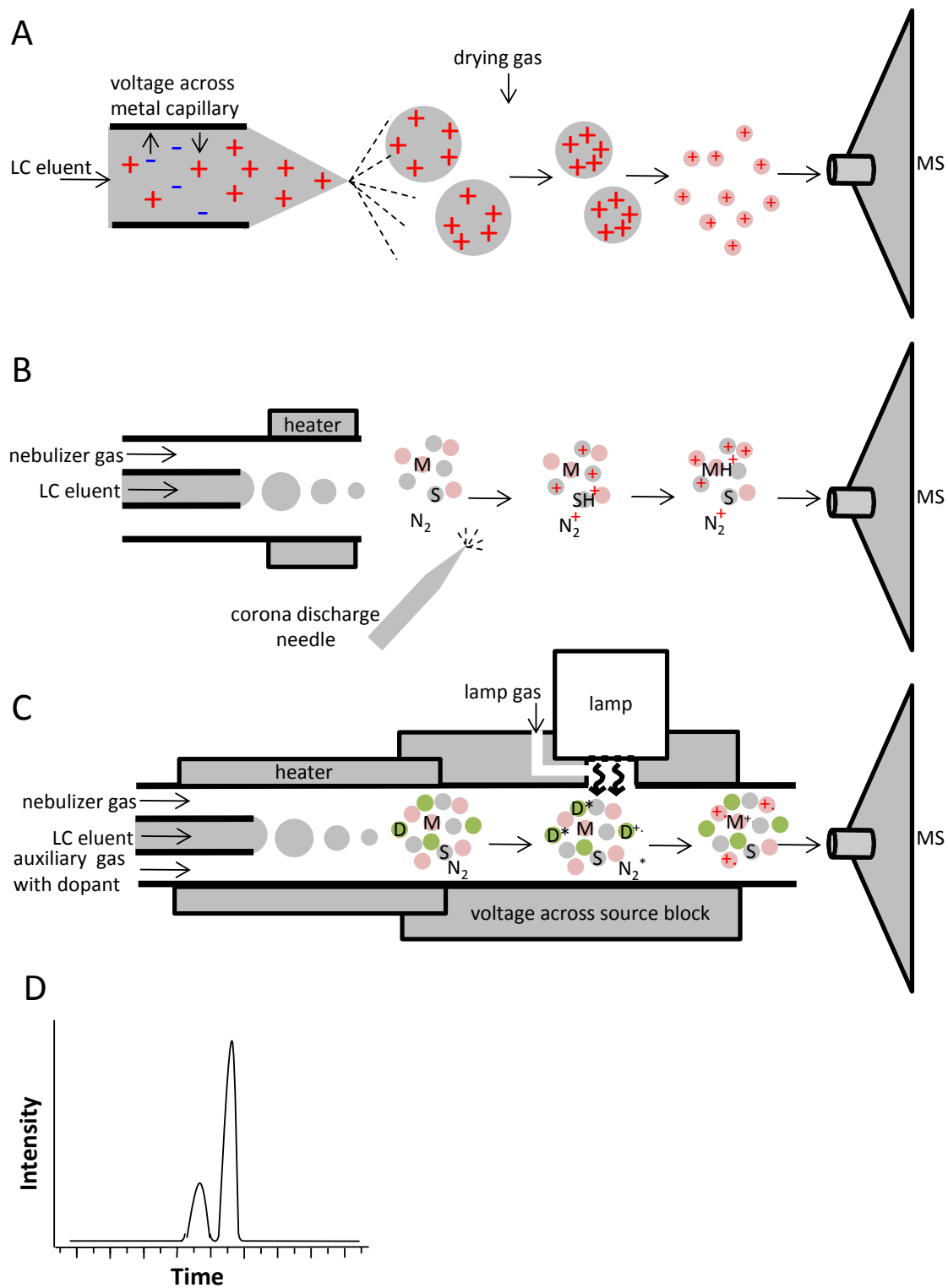


Figure 1.3. ESI, APCI, and APPI ionization sources and example of ionization suppression.

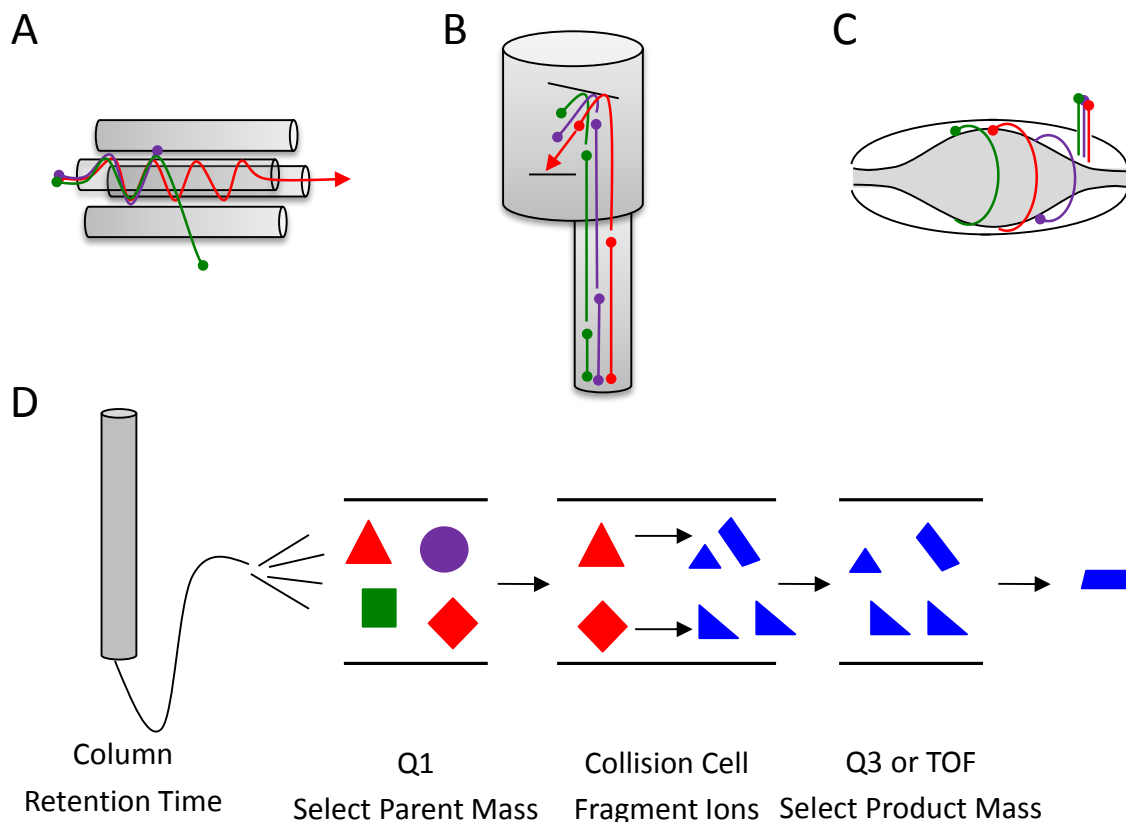


Figure 1.4. Illustrations of A) quadrupole, B) time-of-flight, and C) orbitrap mass analyzers. D) Schematic of MRM experiments.

scan. This list contains a specified parent ion m/z , a collision cell energy and a fragment m/z for a single reaction (SRM), each corresponding to the function of the three quadrupoles. These SRMs commonly make up the library of metabolites for targeted metabolomics experiments.

The number of SRMs in a method is limited by the scan time because the longer it takes to scan through the library the fewer measurements per chromatographic peak. However a decrease in scan time increases the quantitative error in the measurement. One way to address this issue is segmentation of the SRM list in which SRMs are divided into groups by expected retention time. Segmentation requires reproducible retention times, and that the metabolites elute so that their retention times are spaced over several minutes. This is another constraint on chromatographic efficiency by the mass spectrometer. Although SRMs do not contain structural information to the same

extent as an NMR spectrum would, the bond broken in these fragmentation reactions is often the weakest in terms of heterolytic cleavage and can be used to discern differences between isomers. For example, a 2006 article by Rabinowitz and coworkers³⁰ reports that adenosine and deoxyguanosine, although isomers, can be distinguished by SRM because the link between base and sugar breaks in both cases and adenine and guanine are different masses.

Another very popular mass analyzer is time-of-flight (TOF) which separates ions based on the time necessary to reach the detector after each is subjected to the same accelerating force. TOF analyzers have a theoretically infinite mass range (reflectrons $\sim 10,000 m/z$), 10x better mass resolution than a Q, on average 5-10 ppm mass accuracy (although better has been obtained), high sensitivity, and fast scanning times.³¹ The Q-TOF is the instrument of choice for performing untargeted quantitative analyses due to better sensitivity and much higher mass accuracy when compared to a QqQ.

Recently the orbitrap mass analyzer has become more accessible to academic labs, and its use in untargeted metabolomics has already begun.^{24,32} The trap consists of an outer and a central electrode which create an electrostatic field. Ions rotate through this field around the central electrode and are separated based on the frequency of their oscillations along the central electrode.³⁸ The orbitrap has 1-2 ppm mass accuracy and 10x the resolution of a TOF ($\sim 100,000$ resolution) as well as high sensitivity.³² Its advantages and disadvantages over using QqQ analyses for metabolomics analyses has been discussed in detail.³² Here we simply mention the advantages of decreased instrument time necessary for each sample, and better metabolite coverage based on a lack of the constraints on target metabolite library size. The major disadvantage to this analyzer is it, like other trap instruments, is not as quantitative as other techniques. Nonetheless, increased adoption of the orbitrap by the metabolomics field is anticipated as the price continues to decrease.

1.9 Data Processing and Analysis

A series of minimum standards for data analysis and reporting of metabolomics experiments were produced in 2007 by a committee of leaders in the field.³⁹ The denominations used by this committee to describe the series of data treatment steps will be used to outline this discussion of the subject. These standards were originally

developed for one dimensional MS or NMR data. A feature of these types of data is that the signals from all metabolites detected in a given time interval are observed in a single scan and must be deconvoluted, which is one of the common pre-processing steps. Among the techniques used to perform this operation are discriminant analysis, support vector machine learning and principal components analysis, of which the latter is most popular.^{40,41} Although it is possible to apply these methods to two dimensional, tandem MS data sets, they are not necessarily the best methods for analysis of this type of data. This is due to the fact that tandem MS data, especially those using selected reaction monitoring, detect each metabolite in a discrete scan since they separate the analyte of interest from other ions in the first mass analyzer before fragmenting it for specific fragment detection in the final mass analyzer. The data therefore are recorded as “extracted” chromatograms for single metabolites, obviating much of the need for deconvolution.

Such targeted data sets however will nevertheless need peak-picking, peak alignment and target analysis like the one dimensional data sets. Often, small drifts in retention time are observed in large LC sample sets.^{40,42,43} Individual metabolites respond to changes on the column differently, and therefore these shifts are typically not linear in nature or even necessarily in the same direction. Hence, a peak alignment step is necessary for automated handling metabolites consistently across a sample set. For this alignment to be possible, the algorithm must be given an input of peak locations, and a decision is needed on which signals are noise and which belong to a chromatographic peak, otherwise known as peak-picking. Peak integration may also occur at this time. After alignment, target analysis may be completed in which either a region of an NMR spectrum is checked and matched with anticipated peaks, or a particular m/z chromatographic peak is matched with an expected retention time.

The most commonly used software for these pre-processing steps are XCMS⁴⁴ and MzMine,⁴⁵ and these have been compared elsewhere.^{43,46} While a new version of MzMine is in development for two-dimensional data, neither of these programs are currently capable of handling fragmentation data. Proprietary software, like Thermo’s Xcalibur, is useful for data handling; however they may not be deliberately specified for streamlined handling of large datasets like those collected for metabolomics experiments. There are a few open-source programs of varying quality available that build upon the XCMS platform, ie. MzRock, to handle fragmentation data.

Once the data have gone through pre-processing the different data types can be handled much the same way with the exception of flux data which we will discuss in its own section (1.11). The next piece of data handling is data pre-treatment, which can include scaling, centering, log transformation and normalization.³⁹ Pre-treatment would also include any special handling of missing-values and outliers. There are several factors outside the acquired data that may contribute to variability in the data.⁴⁷ These may affect the data in a predictable fashion, such as amount of sample extracted, and changes in ionization efficiency due to partially blocked sample entry. Others factors however may not affect signals predictably, like the person doing the sample preparation, experiment day, gender and inbred line of the subject. The effects of all of these factors should be removed from the data prior to assigning statistical significance and error due to treatment group by normalization.

Processing steps include any statistical model, and the method used to estimate any parameters. Discriminant analysis, support vector machine learning and principal components analysis have all been used for this purpose.^{40,41} Microarray data is processed using a series of linear models, to estimate the contribution of any outside variables, and then the residuals are used to calculate differences between treatment groups.⁴⁸ Both an analogous linear modeling method and simple ANOVA tests of calculated fold-changes have both been used by our lab to determine significance. Post-processing includes back transformation and then data visualization. Validation steps should be continued by training, monitoring and testing the model chosen.

1.10 Data Interpretation

Several systems biology tools can be co-opted for data visualization and interpretation of metabolomics data (Figure 1.4). One of the first ways we visualize any data set is by heat mapping through the open-source Cluster and TreeView programs developed in the Eisen lab.⁴⁹ These programs create an array with the rows denoting a gene (or metabolite) and a column denoting an array (biological sample or sample group). Each block in the array is color coded with the color denoting the direction of change and the color intensity the amount of change. Traditionally red (a hot color)

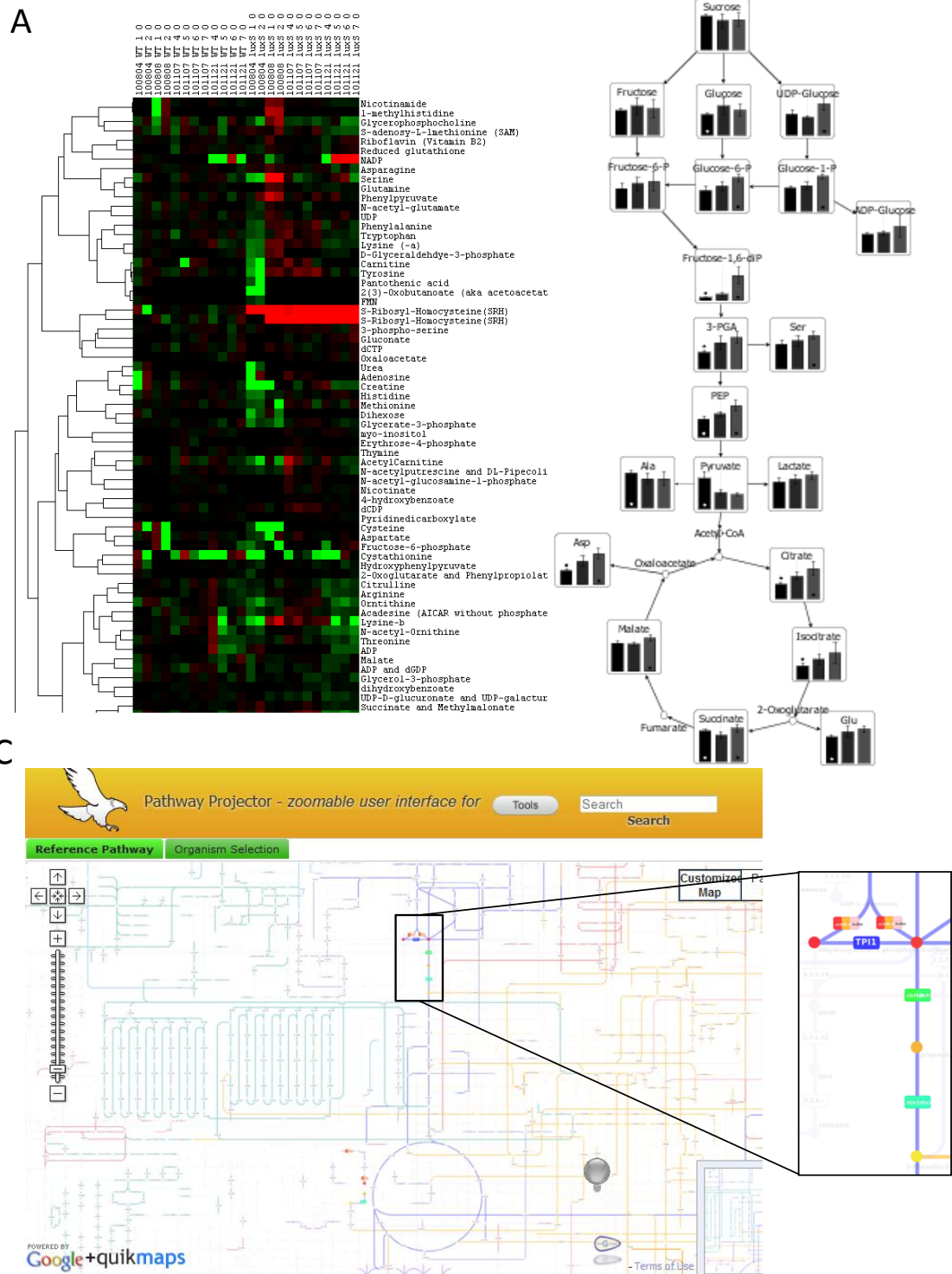


Figure 1.5. Images from A) Cluster, B) VANTED, and C) KEGG pathway projector data visualization programs.

meant an increase when compared to control and green (a cool color) a decrease, thus giving rise to the name “heat map,” although the literature is moving away from these assignments for the benefit of the color blind. These maps visually highlight the largest changes as well as any patterns in the data. The program can also provide information on whether the variation in the data is biological and can be attributed to treatment type or another factor when clustered by “array.” Other visualization programs such as VANTED⁵⁰ can create bar graphs with treatment type or time on the x-axis and overlay them on known metabolic pathway maps. This graphical representation is very useful for spotting changes mid-pathway in response to treatment and can identify the enzyme affected or the point of biosynthetic precursor rerouting. Simpler programs, like KEGG pathway projector,⁵¹ use a list of significantly changed metabolites and they highlight metabolite positions in a global metabolic map and aid in the recognition of relationships between metabolites with changing concentrations.

1.11 The Role of Stable Isotopes in Metabolomics

Stable isotopes are commonly used in metabolomics experiments to either provide a relatively inexpensive source of internal standards or as a way to monitor flux through a biological system. Stable isotope labeled standards are desirable due to the many advantages of using isotope dilution mass spectrometry including controlling for matrix effects with a chemically identical standard and absolute quantitation of metabolites (Figure 1.6 panel A). Unfortunately, labeled standards are prohibitively expensive on the scale of a metabolomics study. Experimental cultures may be grown continuously on stable isotope labeled media to enrich metabolite pools in heavy isotopes.²⁵ Extracts of these cultures may then be spiked with less costly natural-abundance internal standards, before undergoing LC-MS/MS analysis. While this option does save money, maintaining cultures in labeled media can be expensive as well. (¹³C₆)glucose costs about \$60/g and makes about 250 mL of media. The ratio of endogenous metabolite signal to internal standard signal can be used to calculate absolute concentration in the extract (Equation 1.1), although corrections must be made for incomplete labeling. Further measurements and calculations can determine the absolute intracellular concentration of metabolites.²⁵

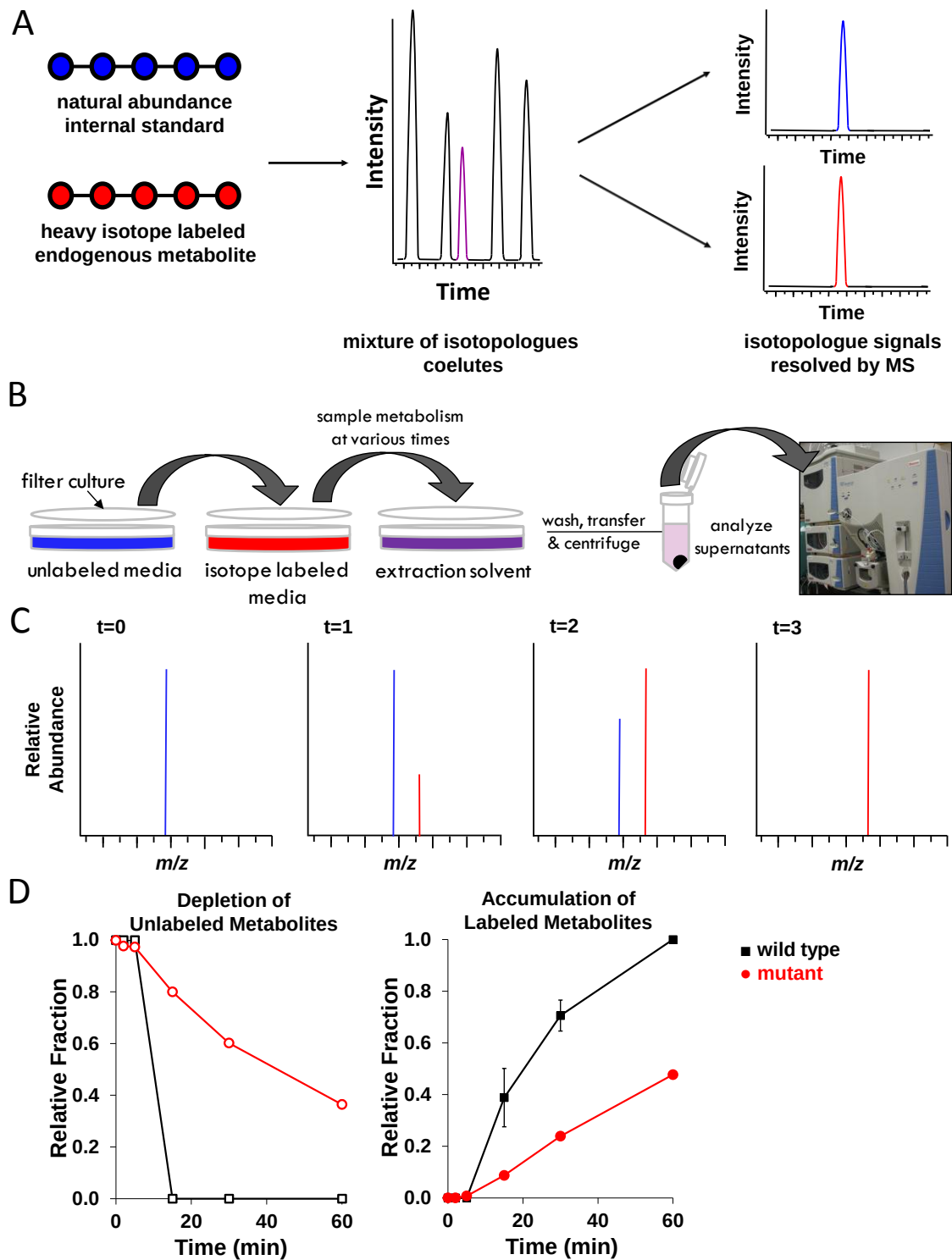


Figure 1.6. Metabolomics related isotope labeling experiments. A) isotope dilution MS experiment. B) Experimental work flow for flux experiments. C) Expected changes in the

mass spectrum of flux samples over time. D) Flux data demonstrating that an enzyme mutation decreases the turn-over rate of its substrate.

Equation 1.1

$$[\text{metabolite}] = \frac{\text{signal isotope labeled endogenous metabolite}}{\text{signal internal standard metabolite}} * \text{known } [\text{standard metabolite}]$$

Besides their utility in isotope dilution mass spectrometry, stable isotopes are useful for tracking the flux of metabolites through a biological system. Kinetic flux profiling (KFP) refers to a technique in which metabolite turnover can be monitored by observation of the incorporation of stable isotopes into metabolite pools over time.⁵² This technique is capable of differentiating between chemically identical metabolites synthesized from distinct sources or at different times.⁵³ KFP studies have proven useful for answering basic science questions such as identifying which similarly functioning pathway is actually being utilized for nitrogen assimilation in *E. coli*.⁵⁴ More translational studies have also benefitted from this technique. Munger and coworkers used KFP as a central discovery tool, and found that human cytomegalovirus and influenza A infection could be slowed by inhibiting the synthesis of certain lipids using commercially available fatty acid synthesis inhibitors.⁵⁵ Another study, reported in 2011, was able to show changes in lipid biology *in vivo*, particularly a triglyceride panel, due to changes in diet.⁵⁶ This study was performed by a group of scientists at Merck and demonstrates the interest the technology has been garnering from commercial industries.

In bacterial culture or cell-lines, KFP is typically performed by either growing the cells in unlabeled media and quickly switching the culture to a labeled media, and monitoring the accumulation of heavy isotope labeled metabolites, or vice versa and monitoring the depletion of heavy isotopes (Figure 1.6).⁵² Cultures are then extracted at several time points and analyzed via MS for isotope incorporation. Perturbations such as treatment with a pharmaceutical or nutrient deprivation may begin before or after the isotope switch. The data are processed by calculating the percent of the total metabolite pool that is labeled. The data can then be graphed to show changes over time and after a model is fit to the data a rate constant is calculated.⁵²

These flux techniques make particularly useful complements to metabolite concentration experiments. Biological systems are particularly good at maintaining

homeostasis,²³ which means metabolite concentrations may not change even though the biosynthetic source or utilization of the metabolite may have changed. Flux experiments can reveal these differences.⁵⁷ When changes in metabolism do occur, an increase in concentration for example may mean either that the organism is making more of the metabolite or that it is using less. Flux experiments can therefore aid in the interpretation of these results by identifying either similar rates of labeling or significantly slower metabolite turn over, respectively.

CHAPTER II

OPTIMIZING OUR METABOLOMICS METHODS

Data presented in the chromatography section has been adapted from the following published journal article:

Jacob N. Fairchild, Krisztian Horvath, Jessica R. Gooding, Shawn R. Campagna and Georges Guiochon. Two-dimensional liquid chromatography/mass spectrometry/mass spectrometry separation of water soluble metabolites. *Journal of Chromatography A* **2010**, 1217, 8161-8166. JNF and KH performed the chromatography experiments and data analysis. JRG prepared the metabolite extracts and together with SRC provided guidance on experimental design based on metabolomics background. JNF, KH and GG provided useful insight on aspects of design and interpretation from the perspective of chromatographers.

Our in-house methods are largely adopted from the work of the Rabinowitz laboratory at Princeton University. The goal of this chapter is to summarize any additions and improvements made or attempted in the process of adapting the method to conditions at the University of Tennessee as well as to changes in the field of metabolomics.

2.1 Optimizing Sample Preparation and Extraction Procedures

Isotopically labeled internal standards and food sources can be expensive, so we sought to introduce a non-biologically synthesized standard that could be used to address technical variability in routine experiments even though it would fall short of absolute quantitation. For this purpose we first determined a rough limit of detection and the four most sensitive SRMs for 3,5-diaminobenzoic acid. The molecule only fragmented one way in negative mode, and this reaction overlapped with the SRM used for xanthine, so another set of internal standards was sought. Benzoic acid was found to work well for negative mode at a concentration of 17 μ M. For positive mode, N,N-dimethylaniline triethylamine and tris(hydroxymethyl)aminomethane (Tris) were tried. Aromatic nitrogen species were found to be less sensitive than their counterparts, because of their relative acidity in a pH= 9.4 buffer. Tris was the most promising of this

set and was chosen for experiments using a concentration 42.5 μM . Representative data in Figure 2.1 illustrates the drop in ions entering the MS over the course experiments reported in chapter 9, as observed by these two internal standards. This data allows us to correct for changes in the MS over time so that concentrations could be compared across samples. Internal standards are added to the extraction solvent prior to its addition to samples, which also enables correction for handling errors.

Published methods²⁵ suggest 80:20 methanol:water for mammalian cells, while acidic 40:40:20 acetonitrile:methanol:water is suggested for gram negative bacteria and yeast. This is a suggestion we have accepted; however, we have tested several extraction solvents for a cyanobacterium, *Prochlorococcus*. Analysis of this bacterium presents several technical difficulties due to its small size (0.5-0.8 μm) and 24 h doubling time. After running a larger panel of extraction solvents, further experiments compared the best two solvents, acidic 40:40:20 acetonitrile:methanol:water¹⁹ and a buffered 80:20 methanol:water with the anti-oxidant ascorbic acid (Kwon),⁵⁸ in triplicate. The results of these experiments highlighted differences in extraction efficiency between the two solvent systems as supported by published findings in *E. coli*¹⁹ (Figure 2.2). The data also supported the continued use of the acidic acetonitrile solvents based on the performance of metabolites relevant to central carbon metabolism such as

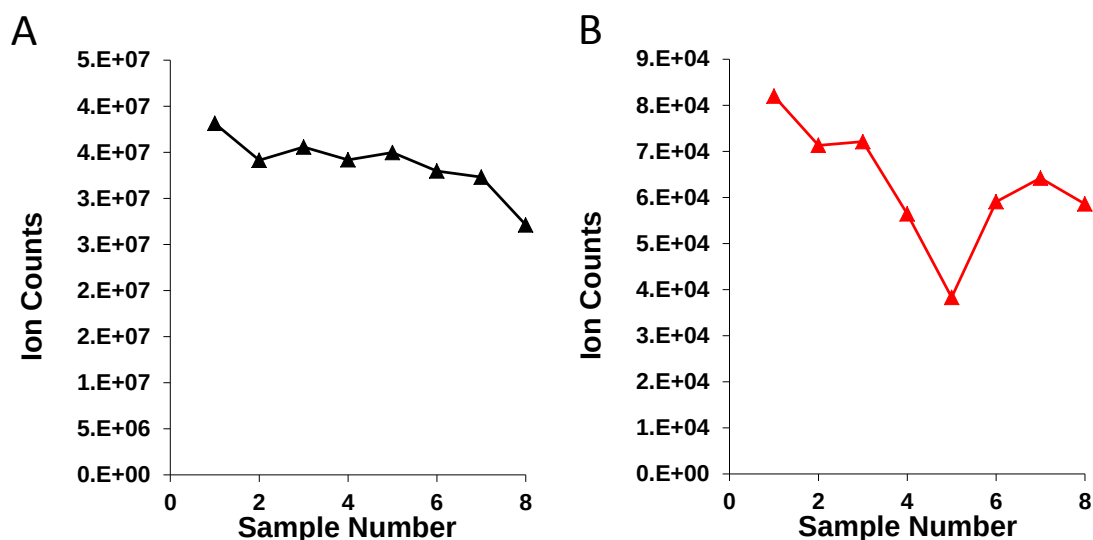


Figure 2.1 Decrease in signal over the course of a metabolomics experiment as observed by A) benzoic acid and B) tris(hydroxymethyl)aminomethane.

the Calvin cycle and pentose phosphate pathway intermediates. In particular, the metabolites phosphoenolpyruvate and PRPP were diminished with the Kwon extraction method when compared to the acidic acetonitrile extraction solvent.

The published methods also suggest performing several extraction steps followed by a final concentration step. Extraction of a large surface area, such as filter cultures and adherent cell culture flasks, requires relatively large volumes to cover all cells. Beyond the initial extraction of an 82 mm filter (2.5 mL), the suggested second rinse adds another 1 mL of volume. Then a second and third extraction of the tiny pellet adds another 100 μ L each. This leads to a total volume of 3.7 mL extracts per sample containing 740 μ L of water.²⁵ If these are then dried, the process will be slow and performed at higher temperatures, leading to degradation of some metabolites. The effect of the additional extraction steps and concentration on *Prochlorococcus* were explored in conjunction with

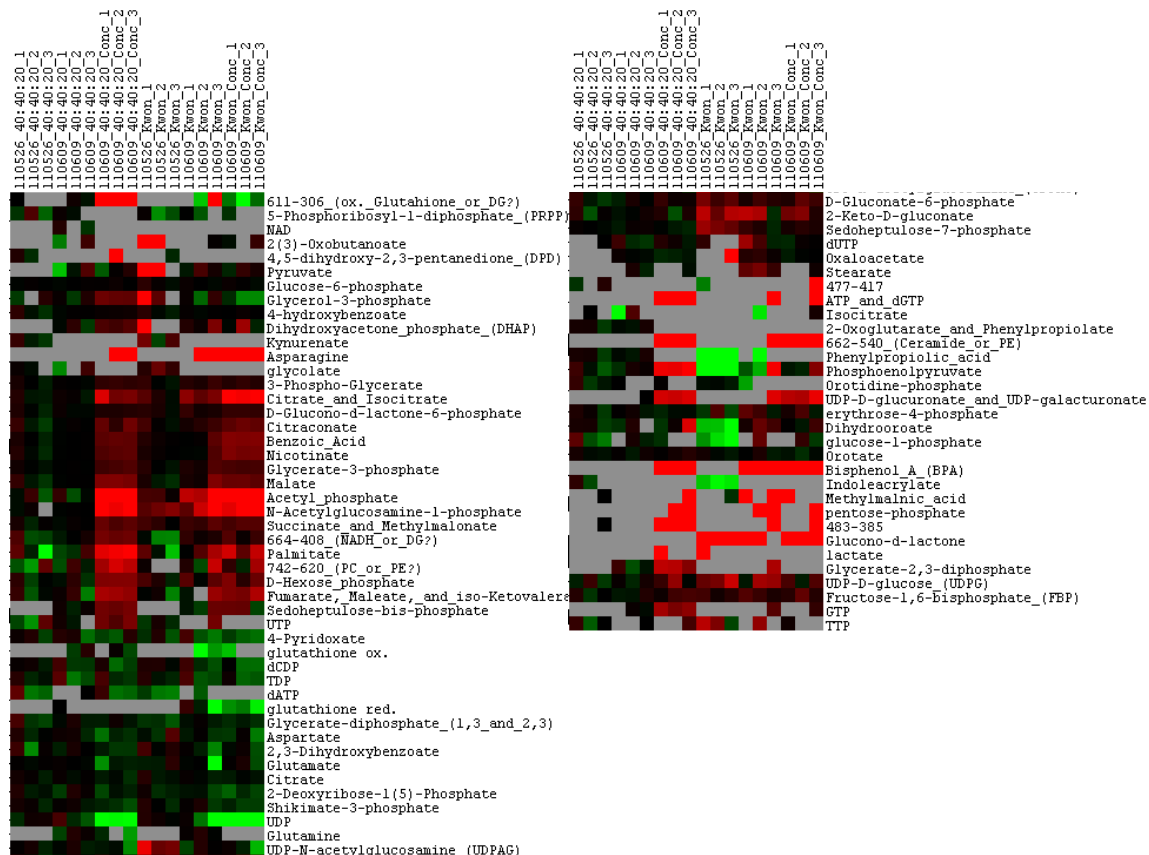


Figure 2.2. Heat map showing relative concentrations of metabolites when extracted with acidic 40:40:20 acetonitrile:methanol:water or with buffered 80:20 methanol:water used by Kwon and coworkers with and without a concentration step.

the solvent experiments described above. While a larger panel of extraction experiments convinced us that few additional metabolites were recovered in the reextraction steps, concentration of the extracted samples was explored in more detail. After drying, solubility issues were observed when the mobile phase was used to reconstitute the sample. We primarily observed an increase in concentration for each metabolite, although degradation was observed for citrate, glycerol-3-phosphate, UDP-glucose, UDP-N-acetylglucoseamine, aspartate, and reduced glutathione. These degraded molecules were deemed essential to the characterization of carbon-flux in *Prochlorococcus* and therefore the repeated extraction and concentration steps were not adopted. A detailed extraction technique will be included for each chapter due to the sample specific nature of the extraction step.

2.2 Changes in LC system

To improve metabolite retention on a reverse-phase column, the Rabinowitz laboratory adopted the use of the ion-pairing reagent tributylamine.^{32,59} Ion-pairing agents can create problems in the other analytical mode (positive mode here) since it competes for ionization and is a major contaminant due to its addition in high concentration to the mobile phase. Tributylamine in particular is difficult to clean from polyether ether ketone (PEEK) tubing and flushing overnight with solvent results in an order of magnitude decrease in the contaminating signal which therefore remains unacceptably high. The Rabinowitz laboratory has a MS dedicated to negative mode to avoid these issues. This solution is an expensive one, so we sought a different LC solution to help us avoid contamination of the whole instrument with tributylamine. A Thermo engineer, Art Flatt, helped us design a custom LC system with two separate pumps and two 6 port valves to avoid contamination. (Figure 2.3). The valve system was also intended to avoid neutralization and washing steps when switching between mobile phases and allow automation when moving between chromatography types. This set up worked in conjunction with autosampler-controlled timed events that allowed automated valve actuation. We soon observed however that the valves were not enough to guard against salt precipitation and clogs in the LC components, especially in the short lengths of shared tubing. Short 20 min washes were introduced prior to the valve switch to

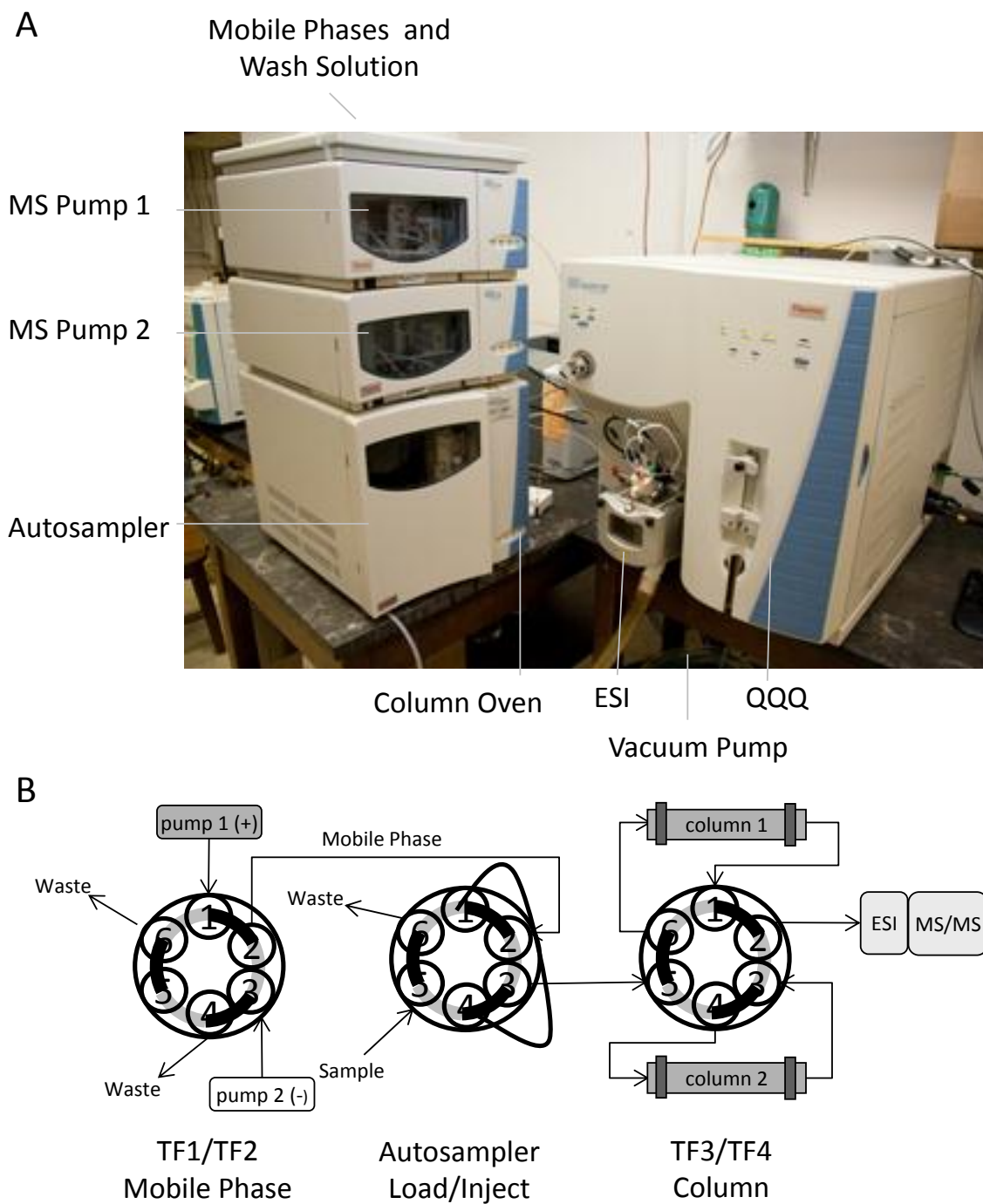


Figure 2.3 Our custom liquid chromatography set up (A), and a schematic of the original automated valve system set up at installation (B).

address this issue; however, to protect the equilibrated columns from non-mobile phase solvents, these washes required manual removal of the columns. These steps improved

the reliability of the system to some extent. However, it was eventually decided that manually changing the tubing took less time than the washing and declogging steps necessary to use the valve system and more robustly prevented clogs and cross contamination with tributylamine. The system was therefore disassembled, but the dedicated second pump has proved a less expensive means of handling the ion pairing agent than the dedication of an entire MS. Removal of the valve system additionally enabled a decrease in the length of tubing between injection and the mass spectrometer which prevents diffusion within the lines. A smaller (0.003) inner diameter tubing was also introduced to prevent diffusion between the injection valve and column and between the column and ESI source.

We do not avoid all problems with the ion pairing agent however. Tributylamine unfortunately sticks to the PEEK tubing and other surfaces in the LC/MS system steadfastly. Our method of switching between pumps prevents introduction of about 1000 fold less tributylamine per positive mode run. However signals observed from contamination and carry-over are still on the order of 10^4 - 10^5 ion counts. These signals are high enough to interfere with mass calibration in positive mode because the instrument mistakes the signal at 186 m/z for the 182 m/z tyrosine standard. To address this point we have simply changed the expected mass to 186.22 which seems to be functional. On the other hand, some of the steps of calibration are surgical in nature, meaning instrument parameters may be adjusted during calibration without notifying the operator or asking for permission. Although these processes can be only poorly understood from the owner's manual, there may be significant reasons not to modify the calibration in this manner that we have not yet diagnosed.

We have had metabolite carry-over issues in the main metabolomics method as well. Two blanks of differing polarity are injected before and after each day of experiments, and the method has been modified to include more stringent washing steps between each injection. A subset of metabolites continues to have comparable signal in the blanks as in the sample. A summary of these metabolites can be found in Table 2.1.

2.3 Two Dimensional Chromatography

The chromatography selected by Bajad and coworkers³⁰ and later updated to include a second method for negative ion mode^{32,59} is as follows. All separations were performed

Table 2.1 Metabolites Often Carried Into Next Sample

Polarity	Compound	Parent <i>m/z</i>	Collision Energy	Product <i>m/z</i>	Expected RT
-	Fumarate, Maleate, and iso-Ketovalerate	115	11	71	24.6
-	Succinate and Methylmalonate	117	10	73	17.4 & 24
-	Citraconate	129	10	85	29
-	Oxaloacetate	131	87	12	25.5
-	4-Hydroxybenzoate	137	21	93	28.6
-	Phenylpropiolate	145	11	101	36
-	2,3-Dihydroxybenzoate	153	17	109	30
-	Orotate	155	111	13	15.6
-	Dihydroorotate	157	12	113	13
-	Glycerol-3-phosphate	171	79	13	13
-	Aconitate (cis and trans)	173.01	15	85	25.8
-	Citrate	191.02	20	87	23.7
-	2-Keto-D-gluconate	193	12	103	12.5
-	Erythrose-4-phosphate	199	97	17	15.4
-	Shikimate-3-phosphate	253	17	97	12.6
-	D-Glucono-d-lactone-6-phosphate	257	20	97	27.5
-	D-Hexose-phosphate	259	79	40	12.1
-	Glucose-1-phosphate	259.01	241	14	17.8
-	Glucose-6-phosphate	259.02	13	199	12.1 & 38.8
-	Glucose-6-phosphate	259.02	199	13	12.1
-	Glycerate-diphosphate (1,3 and 2,3)	265	35	79	37
-	Glycerate-2,3-diphosphate	265.01	12	167	34
-	dCDP	386	25	159	33.1
-	TDP	401	25	159	30.4
-	UDP	403	26	159	27.5
-	664-408 (NADH or DG?)	664	31	408	40
-	Dephospho-CoA	686	35	339	38

Table 2.1 Continued

Polarity	Compound	Parent m/z	Collision Energy	Product m/z	Expected RT
+	Choline	104.01	19	60	13.8
+	Proline	116	11	70	13.3
+	Nicotinamide	123	20	80	7
+	(Iso)Leucine	132	11	86	12.4
+	Aspartate	134	15	74	18.2
+	Glutamate	148	15	84	18.4
+	Carnitine	162	18	103	12.3
+	Acetylcarnitine_DL	204	17	85	11
+	Cytidine	244	12	112	12.1
+	Deoxyadenosine	252	20	136	9.7
+	Glycerophosphocholine	258	14	104	12.6
+	AMP	348.01	21	136	14.2
+	GMP	364	19	152	25
+	5-methyl-tetrahydrofolate	460	25	313.1	23

using a 10 μ L injection of metabolite extract. For those metabolites that ionize most easily by picking up a proton, thereby becoming positively charged, an aminopropyl stationary phase was used (Luna NH₂, Phenomenex, Torrance CA). This 250 x 2 mm column was packed with 100 Å particles with 5 μ m pores and maintained at 10 °C. The mobile phases consisted of solvent A, 95% 20 mM ammonium acetate, 20 mM ammonium hydroxide buffered at pH= 9.4 in HPLC grade water and 5% HPLC grade acetonitrile, and solvent B, HPLC grade acetonitrile. These solvents were used to create a 40 min gradient of the following profile: t) 0 min, 15% solvent A, 85% solvent B; t) 15 min, 100% solvent A, 0% solvent B; t) 28 min, 100% solvent A, 0% solvent B; t) 30, 15% solvent A, 85% solvent B; t) 40, 15% solvent A, 85% solvent B. The flow rate was 150 μ L/min.

For those metabolites that ionize most easily by losing a proton, thereby becoming negatively charged, a C18 stationary phase with a proprietary imbedded polar group was used (Synergi Hydro-RP, Phenomenex, Torrance CA). This 150 x 2 mm column was packed with 80 Å particles with 4 μ m pores and maintained at 25 °C. The mobile phases consisted of solvent A, 11 mM tributylamine and 15 mM acetic acid in 97% HPLC grade water and 3% HPLC grade methanol, and solvent B, HPLC grade methanol. These solvents were used to create a 50 min gradient of the following profile: t) 0 min, 100%

solvent A, 0% solvent B; t) 5 min, 100% solvent A, 0% solvent B; t) 10 min, 80% solvent A, 20% solvent B; t) 15, 80% solvent A, 20% solvent B; t) 30, 35% solvent A, 65% solvent B; t) 33, 5% solvent A, 95% solvent B t) 37, 5% solvent A, 95% solvent B; t) 38, 100% solvent A, 0% solvent B; t) 50, 100% solvent A, 0% solvent B. The flow rate was 200 μ L/min. The eluent was introduced directly into the MS for ion detection.

These methods left room for improvement because chemically related metabolites elute around the same time window, causing ion suppression and making it difficult to segment the SRM library. The column currently used for positive mode likely reacts with cellular components and hence requires frequent replacement. Further the length of the chromatographic runs (40 min positive and 50 min negative) requires 2 h of instrument time per sample and limits the number of samples that can be run in a particular day. The ion pairing agent addressed above (Section 2.2) also creates many technical problems.

An off-line two dimensional chromatography approach could enable crude separation to be completed offline, possibly saving instrument time. However, each extra fraction taken in the first dimension multiplies the number of injections necessary in the second dimension and the number of MS data sets to be analyzed. If fewer fractions are collected, the total volume of each fraction increases. Hence, the metabolite concentration is diluted further, prior to injection in the second dimension.⁶⁰ There is therefore a balancing act when selecting the first dimension method. Because the resolving power of both the second dimension and the mass spectrometer can be relied upon to separate the majority of compounds, a short first dimension, followed by a second workhorse dimension method could be utilized.

Early experiments were completed with a hydrophilic interaction chromatography (HILIC) first dimension using a silicon diol column followed by reverse phase (RP) C18 column with an imbedded polar group for the second dimension. The design was primarily chosen because HILIC and RP are known to be orthogonal.⁶¹ Unfortunately, only poorly resolved peaks were observed for the reverse phase dimension, and so another set of dimensions was pursued.

A strong cation exchange (SCX) column was chosen next for the first dimension. This type of chromatography requires a buffering system, and more traditional buffers would therefore have required a desalting step before MS analysis. To avoid this extra handling step, a volatile ammonium hydroxide – formic acid buffer was used. Of the two

dimensions, HILIC and SCX, the HILIC column could be re-equilibrated fastest, giving us a pragmatic reason for using it second. The first experiments run on this chromatography system used a set of standard metabolite mixtures to determine the necessary length of each run. The first dimension began with 2% of the buffered mobile phase which was increased to 35% over 5 min and held for another 8.5 min. Fractions of 300 μ L were collected equally during this period resulting a total of 18 fractions. The second dimension began with the injection of 50 μ L of a fraction on a silica diol column. The mobile phase started at 15% of 50:50 ACN: 10 mM ammonium formate and was increased to 35% over 2 min, followed by another linear increase to 100% over the next 7 min at 250 μ L/min. This concentration was held 1 min before returning to initial conditions for re-equilibration.

Metabolite extracts from *Escherichia coli* and *Saccharomyces cerevisiae* were then measured using this technique. A total of 141 metabolites were measured, 92 in *E. coli* and 95 in *S. cerevisiae*. The approximate SCX fraction and HILIC elution time for each set of metabolites was plotted in Figures 2.4 and 2.5. The plots demonstrate that the two dimensions are orthogonal. Grouping of chemically similar metabolites can also be observed. For example, the nucleotides cluster around fraction 14 and 10 min, while the small carboxylic acids of the TCA cycle elute around fractions 3-5 and 4 min. While some coelution still occurs, the resolution of metabolites is much improved, and therefore metabolites should be detected more reliably due to prevention of ion suppression. Unfortunately, the method would require 9 h of instrument time to run all fractions in both modes, compared with about 2 h for the Rabinowitz methods. However, if a more targeted method were desired, and a “heart-cut” method were used where only 5-10 fractions were characterized in either positive or negative mode, the method would be 1.25 - 2.5 h per sample and competitive with previous methods. This scenario would be useful in flux experiments where the number of metabolites measured is decreased in exchange for observing isotope labeled metabolite SRMs.^{55,62}

Depending on experimental goals, there is some room for improvement in this method. Metabolite concentrations vary over several orders of magnitude, thus high concentration metabolites should be observed by this method. If however low concentration metabolites were of particular interest, there are a couple options for addressing the dilution problem inherent in two-dimensional chromatography. A larger amount of the metabolite extract solution could be injected in the first dimension, or

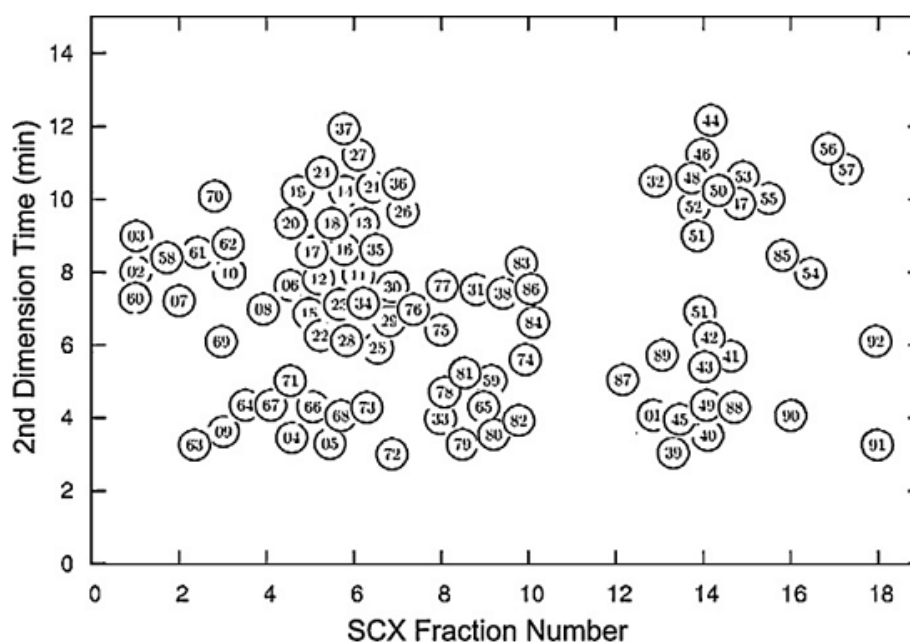


Figure 2.4. *E. coli* metabolites detected in both positive and negative ionization modes. 01-glutamine; 02-lysine; 03-ornithine; 04-aspartate; 05-betaine; 06-betaine aldehyde; 07-o-acetyl-L-serine; 08-urea; 09-proline; 10-thymine; 11-isoleucine; 12-octanoyl-homoserine; 13-acetyl CoA; 14-CoA; 15-cyclic-di-GMP; 16-dephospho CoA; 17-FAD; 18-glutathione; 19-glycerophosphocholine; 20-propionyl CoA; 21-succinyl CoA; 22-TMP; 23-UMP; 24-1-methylhistidine; 25-acetylcarnitine; 26-acetyllysine; 27-arginine; 28-N-carbamyl-aspartate; 29-carnitine; 30-choline; 31-dimethylglycine; 32-histidine; 33-imidazole; 34-imidazole acetic acid; 35-1-methyladenosine; 36-GMP; 37-NADP; 38-creatine; 39-indole; 40-epinephrine; 41-N-acetyl-L-lysine; 42-N-acetyl-glutamine; 43-N-acetyl-glutamate; 44-nicotinamide; 45-pyridoxamine; 46-ADP; 47-AMP; 48-ATP-gamma-S; 49-Biotin; 50-CMP; 51-dAMP; 52-IMP; 53-NAD; 54-serine; 55-dCMP; 56-oxidized glutathione; 57-glucosamine-6-phosphate; 58-2,3-dihydroxybenzoic acid; 59-myo-inositol; 60-orotic acid; 61-UDPG; 62-UDPG; 63-aconitate; 64-isoketovalerate; 65-maleic acid; 66-fumarate; 67-malate; 68-phenylpyruvate; 69-phosphoenolpyruvate; 70-dephospho-CoA; 71-kynurenine acid; 72-4-hydroxybenzoic acid; 73-lactate; 74-citrate; 75-d-glyceraldehyde-3-phosphate; 76-DHAP; 77-glycerol-phosphate; 78-glucono-lactone; 79-isocitrate; 80-pantothenic acid; 81-glyceric acid; 82-oxaloacetate; 83-cAMP; 84-pyridine-dicarboxylate; 85-glucose-1-phosphate; 86-succinyl/methylmalonyl CoA; 87-xanthine; 88-nicotinate; 89-inosine; 90-hypoxanthine; 91-fructose-bis-phosphate; 92-gluconate.

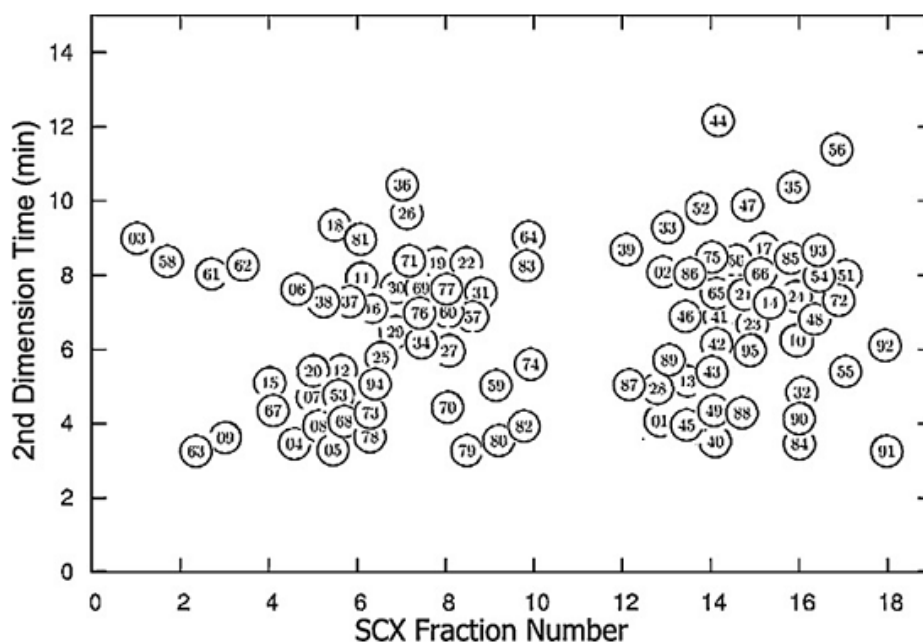


Figure 2.5. Yeast metabolites detected in both positive and negative ionization modes. 01-glutamine; 02-4,5-dihydroxy-2,3-pentanedione; 03-ornithine; 04-aspartate; 05-betaine; 06-betaine aldehyde; 07-2-oxoglutarate; 08-4-hydroxybenzoate; 09-proline; 10-adenosine; 11-iso-leucine; 12-allantoate; 13-5'-methylthioadenosine; 14-asparagine; 15-acetyl-CoA; 16-adenine; 17-alanine; 18-glutathione; 19-cytidine; 20-carbamoyl aspartate; 21-cytosine; 22-d-hexose-phosphate; 23-dihexose; 24-citrulline; 25-acetylcarnitine; 26-acetyllysine; 27-d-glyceraldehyde-3-phosphate; 28-folate; 29-carnitine; 30-choline; 31-dimethylglycine; 32-DL-pipecoline; 33-glucosamine; 34-erythrose-4-phosphate 35-flavone; 36-GMP; 37-glucose-6-phosphate; 38-fructose-6-phosphate 39-GABA; 40-epinephrine; 41-N-acetyl-L-lysine; 42-N-acetyl-glutamine; 43-N-acetyl-glutamate; 44-nicotinamide 45-pyridoxine; 46-glutamate; 47-AMP; 48-guanosine; 49-biotin; 50-methionine; 51-phenylalanine; 52-IMP; 53-phenylpropionate; 54-serine; 55-riboflavin; 56-oxidized glutathione; 57-methylcysteine; 58-methylmalonate; 59-myoinositol; 60-N-acetylglucosamine-1-phosphate; 61-UDPAG; 62-UDPG; 63-aconitate; 64-NAD; 65-N-carbamoyl-L-aspartate; 66-nicotinamide ribotide; 67-malate; 68-phenylpyruvate; 69-sedoheptulose-7-phosphate; 70-succinate; 71-trehalose-6-phosphate; 72-typtophan; 73-lactate; 74-citrate; 75-tyrosine; 76-DHAP; 77-glycerol-3-phosphate; 78-pyruvate; 79-isocitrate; 80-pantothenate; 81-S-adenosyl-L-homocystein; 82-oxaloacetate; 83-cAMP; 84-S-ribosyl-homocysteine; 85-glucose-1-phosphate; 86-threonine; 87-xanthine; 88-nicotinate; 89-inosine; 90-hypoxanthine; 91-fructose-1,6-bis phosphate; 92-gluconate; 93-valine; 94-UTP; 95-xanthosine.

more, smaller fractions could be collected. If reducing analysis time however is the main goal, fraction number could be reduced at the risk of detecting fewer metabolites. A better option would be reducing the diameter of the second column. This option would allow a comparatively faster flow rate and improve ionization efficiency thus reducing the time necessary for each analysis (probably by half) and increase the sensitivity at the same time.^{63,64} We did not have a smaller column to test this option in more detail. Other options for decreasing the time necessary for analysis include optimization for a select subset of metabolites and reverting to a one dimensional analysis based on the diol HILIC second dimension. This final option loses the resolution available via two-dimensional chromatography, but would still allow a single separation method to be shared between positive and negative modes, eliminate the need for tributylamine, and eliminate the use of a reactive amino propyl column.

In the end however, we chose to continue using the Rabinowitz method for routine analyses because the new method provided only incremental improvement upon global metabolomics methods. This choice also avoided much work validating the identity of all library metabolites and resegmenting the SRMs appropriately.

2.4 Adjustments and Additions to Mass Spectrometry Methods

After separation, the eluent is directly introduced into the ESI source through a 0.1 mm internal diameter fused silica capillary. The spray voltage was set to 4500 V for positive ion detection and 3000 V for negative ion detection. The inlet capillary temperature was set to 290 °C and the nitrogen sheath gas was set to 40 psi. The ions then entered a Finnigan TSQ Quantum Discovery Max triple quadrupole mass spectrometer (Figure 2.3) where they were detected using multiple reaction monitoring mode. The argon collision gas pressure was set to 1.5 mTorr. The scan time for each SRM was 0.05 s and the scan width was 1 *m/z*. The detection parameters for each metabolite can be found in Table A2.1 and Table A2.2. Those metabolites added by our lab are in italics, and those standards tested in our lab are underlined. Primarily, those added by our lab are markers of oxidative stress, those related to quorum sensing, and some sugar phosphates. Some re-segmenting has been completed to ensure that no metabolite peaks have been missed due to differences in metabolite matrix effects and chromatography between labs. We have in particular noticed that high salt content

samples, such as our studies in marine environments, tend to exhibit significant changes in retention time. Experiments to build a method suitable for these high salt samples are planned.

Some of these SRMs are not perfectly selective. A set of isomers for example may both produce a fragment of the same mass, but they may be retained for different lengths of time or undergo the fragmentation reaction at different efficiencies. The isomers may then be differentiated despite these imperfections. Some of the known SRM overlaps are listed in Table A2.3.

2.5 Evolution of Data Analysis

The two major difficulties with data analysis for our methods seem to lie in using a two dimensional MS method, and the type of raw data file (.RAW) recorded. These each prevent the use of popular metabolomics data handling programs, which then requires the use of less robust peak picking, peak integration and peak alignment software and a flexibility to adapt new software programs as they are released. We used the program mzRock for some time; however the lack of flexibility in the software to correct false negatives led us to turn to the software package Xcalibur (Thermo Electron, Waltham MA). Xcalibur Quan Browser is used to pick and calculate integrations for all peaks matching our library. All peaks are then manually curated to ensure peaks are called consistently and no false positives or false negatives are introduced by the computer. This data set can then be exported to Excel (Microsoft, Redmond WA), where it undergoes normalization, statistical analysis, and fold change calculations. The development of our own program for normalization and statistical analysis based on linear modeling is underway. The data is then processed by one of several visualization programs, the first of which is Cluster for heat mapping.

2.6 Conclusions

We have taken a previously described method and tried several means of optimizing it. To this end, a second pump was installed to prevent the contamination of the entire LC-MS/MS with tributylamine. Extraction methods were optimized for a new cyanobacteria, *Prochlorococcus*, and concentration was determined to be detrimental to the samples. A new set of internal standards have also been introduced in order to

correct for changes in the MS using a less expensive means. Further, several new compounds have been added to the SRM library, including those relevant for photosynthetic organisms and several stress markers. The use of this method will be described in several secondary projects as a means of illustrating their actual and potential impact.

2.7 Methods

General protocol for the addition of a new metabolite. Standards were dissolved to a concentration of ~ 1 mg/mL in methanol or water. Serial dilutions were made to 1 µg/mL in methanol. This solution was directly infused into the MS at a flow rate of 10 µL/min and the concentration was increased until a strong signal was observed. The compound optimization menu in Xcalibur was used to automatically find the four most sensitive SRMs for the parent mass in question. The molecule was then run through the appropriate chromatography method multiple times and retention time was assigned. This information was then added to the appropriate segment of the standard method.

Optimization of Prochlorococcus Extraction. See Chapter 4.

2D-LC Materials. HPLC grade water and acetonitrile as well as reagent grade ammonium hydroxide were purchased from Fisher Scientific (Pittsburgh, PA). Reagent grade ammonium formate and LC/MS grade formic acid were obtained through Sigma-Aldrich (St. Louis, MO). The first dimension column used was a 150mm × 4.6mm Luna SCX (Phenomenex, Torrance, CA). This column consists of fully porous, 5 µm particles with bonded aryl-SO₃⁻ groups. The second dimension column was a 150mm × 3.0mm Luna HILIC (Phenomenex). The packing material of the HILIC column consisted of 3 µm fully porous silica diol particles.

2D-LC Microbial strains and growth conditions. Wild type *E. coli* strain BW25113 was purchased from the Coli Genetic Stock Center at Yale University. Cultures of *E. coli* were grown in minimal media at 37 °C with aeration until to mid to late exponential phase (Optical Density at 600 nm (OD₆₀₀) = 0.915 at the time of extraction). *S. cerevisiae* was

purchased from Carolina Biological (Burlington, NC) and grown in Yeast Mold Broth at 30 °C with aeration to an OD₆₀₀ of 0.373 before extraction.

2D-LC Metabolite extraction. Metabolites were extracted from *E. coli* and *S. cerevisiae* cells similar to the method described by Bajad et al.³⁰ and Lu et al.³² For the *E. coli*, a 48 mL culture was centrifuged (6 min, 3 x10³ rcf, 4 °C) to concentrate the bacteria cells. The supernatant was discarded and the pellet was resuspended in extraction solvent (1300 µL, 2:2:1 ACN:MeOH:H₂O with 0.1 M formic acid). Due to added complications caused by the numerous metabolites in rich media, *S. cerevisiae* cultures were vacuum filtered directly onto a nylon filter (Part #: R04SP04700, GE Osmonics, Minnetonka, MN) before extraction to remove excess media and to collect the cells. The filter was then immediately placed face down in a petri dish containing 1300 µL of extraction solvent to sample the metabolome. Both microorganisms were allowed to incubate in the extraction solvent for 15 min at 4 °C. For *S. cerevisiae* samples, the filters were then rinsed with the previously added extraction solvent by pipetting a steady stream over the surface of the filter for about 2 min to resuspend the cells in the extraction solution. After incubation, the suspensions containing the metabolites were transferred to 1.5 mL centrifuge tubes, and any remaining cell debris was removed by centrifugation (5 min, 16.1 x10³ rcf, 4 °C). An 840 µL aliquot of the supernatant was mixed with 72 µL of 15% bicarbonate (to neutralize the solution), 5 µL of 4.25 mM Tris and 5 µL of 1.7mM benzoic acid (here benzoic acid and Tris are added as internal standards). The extracts were stored at -80 °C until use.

2D-LC Instrumentation and data handling. Experiments were performed on a Thermo Finnigan (San Jose, CA) Surveyor liquid chromatograph equipped with a quaternary pump, autosampler, and convective column oven. The chromatograph was connected to a TSQ Quantum Discovery Max (Thermo Finnigan, Waltham MA) triple quadrupole mass spectrometer for detection. Both the chromatograph and mass spectrometer were controlled simultaneously by Thermo's Xcalibur 2.0 software. Eluates were introduced into the electrospray ionization (ESI) chamber through a 0.1mm I.D. fused silica capillary. Mass spectrometry methods were the standard protocols described above.

2D-LC Experimental setup. In the first dimension, a 100 μL volume of the sample mixture was injected onto the strong cation exchanger column. The mobile phases for the cation exchange gradient separation were (mobile phase A) 75:25 $\text{H}_2\text{O}:\text{ACN}$ and (mobile phase B) 75:25 400 mM $\text{NH}_4\text{HCO}_2:\text{ACN}$. The volatile buffer, 400 mM NH_4HCO_2 (ammonium hydroxide-formic acid buffer) was prepared by titrating 400 mM ammonium hydroxide to pH 3.25 with formic acid. Since the pK_a of formic acid is 3.75, only 25% of the formic acid is deprotonated and combined with the ammonium cation, the total cationic strength of this buffer is 725 mM. For the cation exchange gradient, mobile phase B was increased linearly from 2–35% in 5 min and held at 35% until all components were eluted with a constant flowrate of 0.4 mL/min. The fractions from the first dimension were collected using a Gilson F203 fraction collector (Gilson, Middleton, WI, USA), retrofitted with F203b internal hardware. Eighteen equal volume fractions were collected from 2.5 to 8.5 min across the first dimension. Fractions were collected in 300 μL vials and loaded into the autosampler quickly, where they were chilled to 4 $^\circ\text{C}$ until injection. For the second dimension, 50 μL injections were made onto the silica diol column. The HILIC separation the mobile phases were: A = 95:5 $\text{ACN}:100$ mM ammonium formate, pH 3.2, and B = 50:50 $\text{ACN}:10$ mM ammonium formate, pH 3.2. This mobile phase system was chosen to provide a constant 5 mM concentration of ammonium formate throughout the second dimension separation. The second dimension gradient consisted of two linear increases of mobile phase B operated at a constant flow rate of 0.25 mL/min. The gradient was initially increased from 15–35% mobile phase B in the first 2 min, followed by an increase from 35–100% mobile phase B from 2–9 min. Mobile phase B was held at 100% for 1 min then returned to the initial conditions for re-equilibration of the column.

CHAPTER III

RIBONEOGENESIS IN YEAST

Data presented in this chapter has been adapted from the following published journal article:

Michelle F. Clasquin, Eugene Melamud, Alexander Singer, Jessica R. Gooding, Xiaohui Xu, Aiping Dong, Hong Cui, Shawn R. Campagna, Alexei Savchenko, Alexander F. Yakunin, Joshua D. Rabinowitz, and Amy A. Caudy. Riboneogenesis in Yeast. *Cell* **2011**, 145, 969-980. Members of the Princeton Departments of Chemistry and Lewis-Sigler Institute for Integrative Genomics (MFC, EM, SRC, JDR, AAC) completed the discovery metabolite profiling and pathway identification. Those authors at the University of Toronto completed the X-ray crystallography. JRG prepared and characterized the heptulose and octulose phosphates.

3.1 Introduction

Eukaryotic cells consume glucose by two major pathways, glycolysis and the pentose phosphate pathway (PPP). Glycolysis produces 2 units of ATP, 2 units of NADH, and trioses for the synthesis of amino acids and lipids. The pathway is regulated in four ways. Most hexokinase isozymes are allosterically inhibited by the product glucose-6-phosphate. Phosphofructokinase is also allosterically inhibited by products of glycolysis, namely ATP, citrate, and pH (modified by increased lactic acid levels). Pyruvate kinase is activated by an upstream product of glycolysis fructose-1,6-bisphosphate. Notably, these three steps are irreversible, and two of these reactions are also used to control the net direction of flux through the pathway (glycolysis or gluconeogenesis).

The PPP can be broken into two portions: the oxidative stage and non-oxidative stage. The oxidative PPP produces 2 NADPH, ribulose-5-phosphate, and carbon dioxide. Ribulose-5-phosphate (Ru5P) can then be entirely converted to ribose-5-phosphate (R5P), which is necessary for the synthesis of nucleic acids or converted into glycolysis intermediates via the non-oxidative reactions. These glycolysis intermediates can be recycled back into the oxidative PPP via gluconeogenesis or used to produce ATP, NADH, and trioses. The oxidative stage is regulated by NADPH inhibition of the

first step which converts glucose-6-phosphate to gluconolactone-6-phosphate. This enables regulation of glucose-6-phosphate catabolism that is dependent on the presence of products of both pathways. Remarkably, the non-oxidative reactions are largely considered to be in equilibrium and performed by promiscuous enzymes. No enzyme regulation within the pathway is known to be capable of directing carbon flow either towards ribose-5-phosphate synthesis or towards glycolysis intermediates. The textbook version of the non-oxidative steps are debated and arguably cannot explain all isotopic distributions observed,⁶⁵⁻⁶⁷ leaving the possibility open for other currently unknown steps. The non-oxidative steps of the PPP, despite being poorly understood, are evidently important, since the activity of the constitutive enzymes must be upregulated for the growth of many tumor cells.⁶⁸

In the model organism, *S. cerevisiae*, more than 900 genes have unknown functions according to the Saccharomyces Genome Database. Current databases of sequence, transcription, and proteomic information allow these >900 to be narrowed to putative enzymes that are largely expressed and conserved. These candidates can then be characterized via discovery metabolite profiling²² and assigned function with further validation. These experiments⁶⁹ have previously shown that in vitro activity measurements are not adequate for the prediction of endogenous enzyme substrates. Here we assign sedoheptulose-1,7-bisphosphatase activity to the previously uncharacterized gene YKR043c, a thermodynamically driven route to ribose production that does not require NADPH production.

3.2 SBP and OBP accumulate in *S. cerevisiae* *shb17* mutant

A metabolomic screen of single deletion mutants of genes with unknown functions was started. Candidates were selected based on whether they contained protein domains similar to known enzymes. Metabolic phenotypes of each were then compared to the wild type strain using an untargeted approach by separating extracts on a reverse-phase column then analyzing the eluent by high resolution orbitrap mass spectrometer.

When the gene YKR043c, which we have renamed *shb17*, was deleted, four metabolites accumulated and another depleted when compared to the wild type. Exact masses of each of the five indicated this was a set of mono- and bisphosphorylated seven and eight carbon sugars (Figure 3.1). This conclusion was further supported by

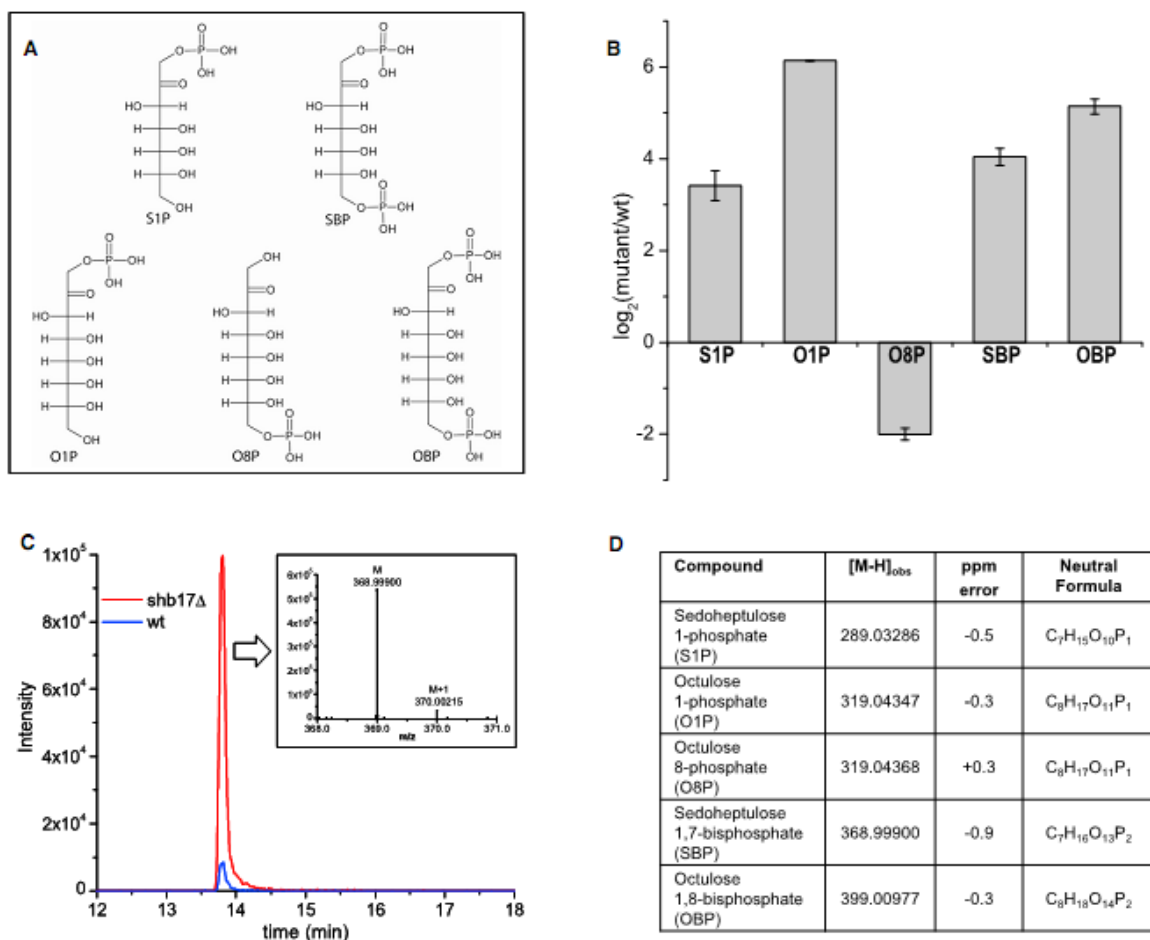


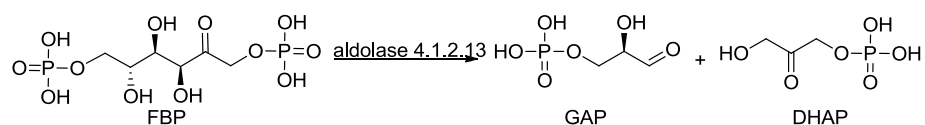
Figure 3.1 Characterization of the $\Delta shb17$ metabolic phenotype. A) Structures of the metabolites associated with the $\Delta shb17$ phenotype. B) Relative concentrations of metabolites. Data are the average of 4 biological replicates $\pm$ SE. C) The extracted ion chromatogram for SBP in $\Delta shb17$ and wild-type strains and mass spectrum displaying the accurate mass for the parent ion. (D) Table of accurate masses for $[M-H]$ ions.

labeling studies. When the $\Delta shb17$ cultures were fed ($^{13}\text{C}_6$)glucose a mass change of +7 or 8 m/z was observed respectively. No change was observed when yeast were labeled with $^{15}\text{NH}_4\text{Cl}$. Fragmentation of each resulted in a $[\text{H}_2\text{PO}_4]^-$ peak. Seven-carbon bisphosphates had been included in metabolic databases, but were not previously thought to occur in yeast. Eight-carbon sugar phosphates were not included in common databases but were described in older literature.⁷⁰⁻⁷² We hypothesized that those accumulated were sedoheptulose-1-phosphate (S1P), sedoheptulose-1,7-bisphosphate (SBP), octulose-1-phosphate (O1P) and octulose-1,8-bisphosphate (OBP) and the depleted one was octulose-8-phosphate (O8P).

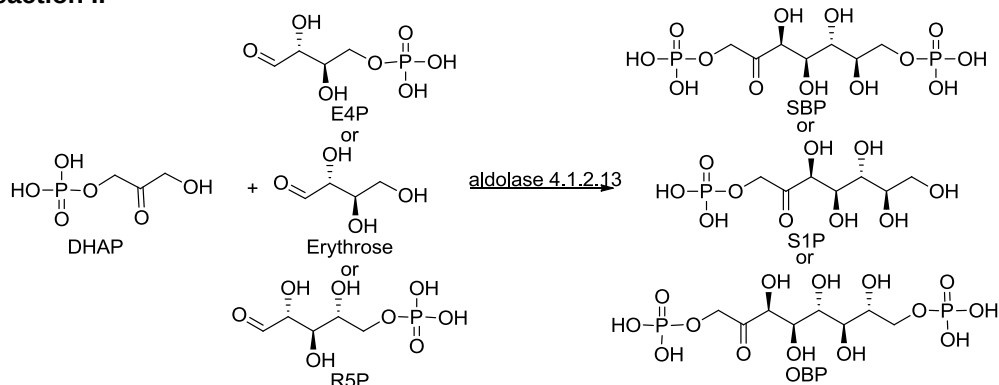
3.3 Synthesis of SBP, S1P and OBP

Three of these sugars were synthesized in order to compare standards to those signals observed in yeast extracts and to enable further kinetic and structural studies. A series of coupled enzymatic reactions (Figure 3.2) were used to produce sedoheptulose-1,7-bisphosphate (SBP), sedoheptulose-1-phosphate (S1P), and *D-glycero-D-altro*-octulose-1,8-bisphosphate (OBP). In Reaction I, aldolase (4.1.2.13) cleaves fructose-1,6-bisphosphate (FBP) into glyceraldehyde-3-phosphate (GAP) and dihydroxyacetone-phosphate (DHAP). Reaction II uses aldolase (4.1.2.13) to couple DHAP to erythrose-4-phosphate (E4P), erythrose, or ribose-5-phosphate (R5P) to form the products SBP, S1P, or OBP, respectively. In the case of SBP, erythrose-4-phosphate is derived from the cleavage of fructose-6-phosphate by transketolase (2.2.1.1) in the presence of GAP, which produces xylulose-5-phosphate as a side product. Finally in Reaction III, triose phosphate isomerase (5.3.1.1) is used to facilitate conversion of DHAP to GAP, thus driving the reaction and increasing yield. These methods are modifications of previous work.^{73,74} Both *D-glycero-D-altro*-octulose-1,8-bisphosphate and *D-glycero-D-ido*-octulose-1,8-bisphosphate are thought to be biologically active. The *altro* form was synthesized first for comparison because it could be synthesized by the methods described. Arabinose-5-phosphate, which would be required for the synthesis of the *ido* form, was much more expensive. These standards did match retention times and fragmentation observed for endogenous compounds. O1P and O8P were not synthesized, but comparison of each to S1P and S7P supported assignment of the site of phosphorylation (Figure 3.3).

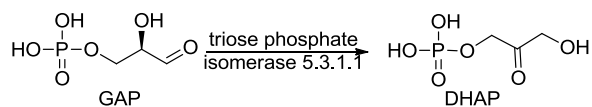
Reaction I



Reaction II



Reaction III



Source of E4P

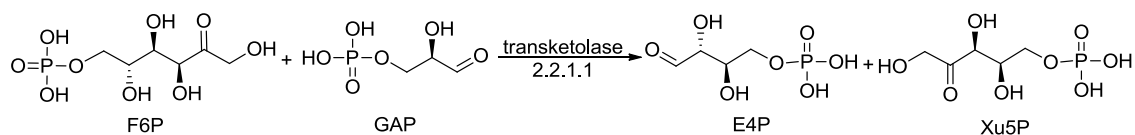


Figure 3.2 The series of enzymatic reactions used to synthesize SBP, S1P and OBP.

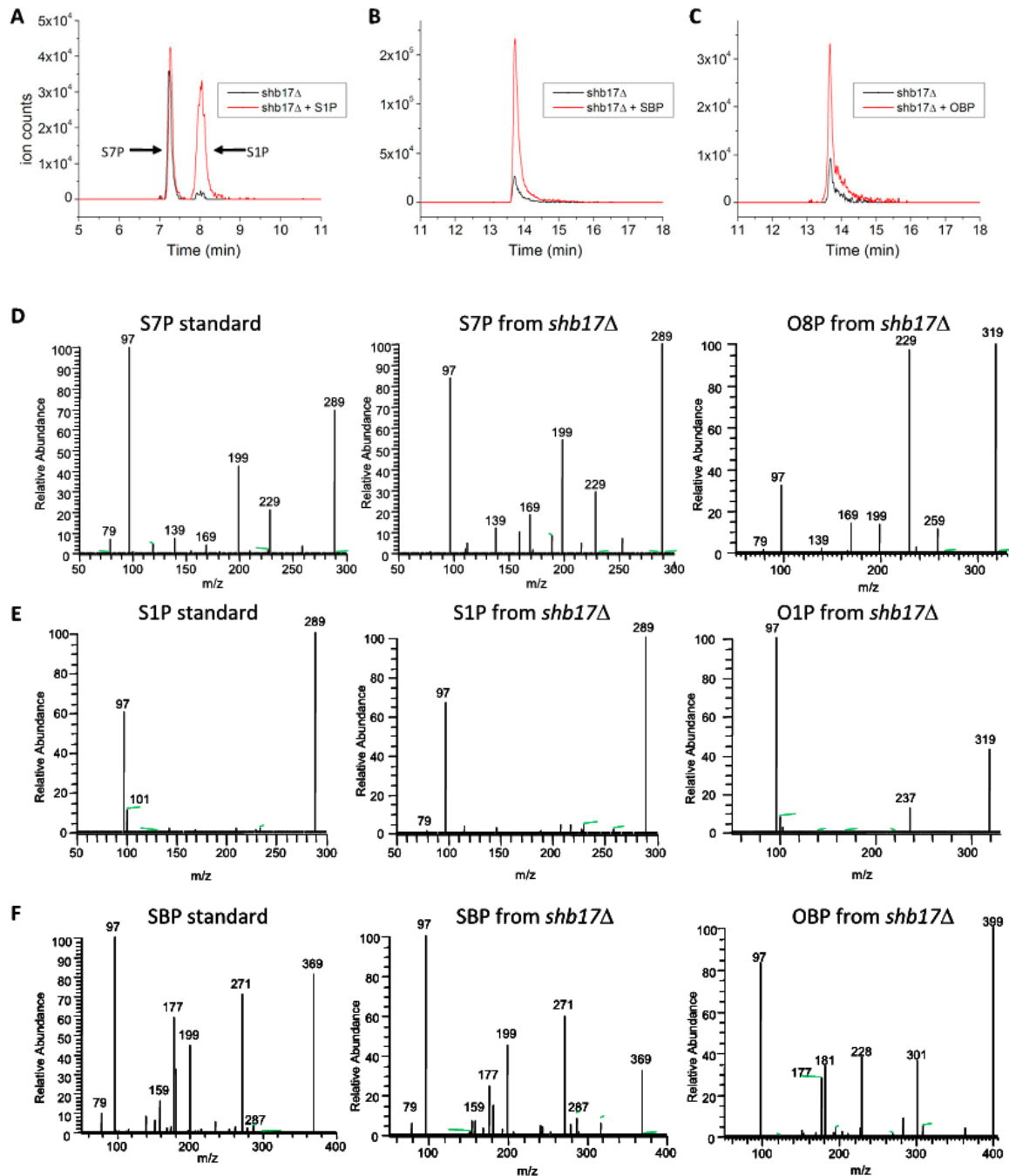


Figure 3.3. Confirmation of metabolite identity through synthetic standards. A-C) Chromatograms showing retention time match between the metabolites measured in $\Delta shb17$ and synthetic standards. D-F) Product ion spectra acquired for the synthetic standards of S7P, S1P, and SBP and compared to the sedoheptulose and octulose metabolites those from $\Delta shb17$. Note the octuloses fragment in the same manner as the heptuloses.

3.4 Shb17 is a selective sedoheptulose and octulose biphosphatase

In order to determine the substrate specificity of Shb17, recombinant enzyme was incubated with the candidate substrates S1P, SBP and OBP. Cleavage of SBP and OBP to S7P and OBP was observed, but Shb17 had no effect on S1P. This phosphatase activity was inhibited by vanadate, and no phosphate transfer to AMP or ADP was observed. It was previously reported that *shb17* also demonstrated phosphatase activity on FBP.⁷⁵

Although Shb17 does cleave FBP, there was no difference observed in FBP concentration between mutant and wild type cultures when measured by an isotope ratio based approach²⁵ (1.033 mutant/wt, p=0.6). When SBP or FBP incubation with Shb17 was monitored (Figure 3.4), the enzyme was found to be more selective for SBP ($K_M = 34 \pm 8 \mu\text{M}$, $v_{\max} = 7.8 \pm 0.7 \mu\text{mol/min/mg protein}$). Intracellular concentrations of FBP ($K_M = 510 \pm 50$, $v_{\max} = 2.6 \pm 0.1$) are 100x that of SBP when measured by an isotope ratio based method,²⁵ indicating FBP could compete for enzyme sites more effectively but the overall concentration of FBP is less likely to be effected by phosphatase activity. Velocity of a reaction where two substrates are competing for the same enzyme can be described by equation 3.1.

Equation 3.1

$$v_t = \frac{\left(\frac{V_{\text{SBP}}}{K_{\text{SBP}}}\right)[\text{SBP}] + \left(\frac{V_{\text{FBP}}}{K_{\text{FBP}}}\right)[\text{FBP}]}{1 + \frac{[\text{SBP}]}{K_{\text{SBP}}} + \frac{[\text{FBP}]}{K_{\text{FBP}}}}$$

Using the intracellular concentrations, typical cell volume, $v_{\max}$ and K_M , the overall rate of phosphate cleavage can be calculated. On the whole, 6% of the total SBP concentration undergoes Shb17 hydrolysis per minute, whereas 0.16% of the total FBP pool is cleaved. Therefore, sedoheptulose-1,7-bisphosphatase and possibly octulose-1,8-bisphosphatase activity is likely the primary function of Shb17 *in vivo*.

3.5 The Shb17-SBP complex structure

Recently a crystal structure of an inactive shb17 containing a mutation of histidine 13 to alanine (H13A) was reported⁷⁵ with FBP in the active site. The FBP was found to bind linearly, not as the thermodynamically more stable cyclic furanose typically observed in

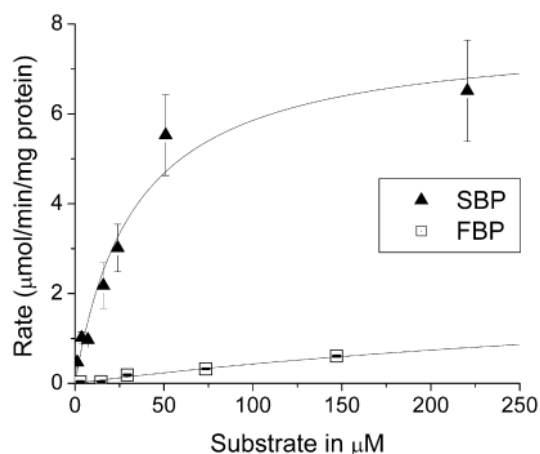


Figure 3.4. Reaction kinetics of hydrolysis of SBP and FBP by recombinant Shb17. Data are the average of two independent experiments $\pm$ standard error.

other FBP binding enzymes. We co-crystallized SBP with Shb17 (H13A) and the structure was solved to a 2.2Å resolution (Figure 3.5).

The Shb17 protein is a dimer, with a series of α -helices surrounding a central β -sheet. The ligand binding site is on one side of the central β -sheet in a pocket defined by the α 1, α 7, α 8, and α 9 helices as well as their intervening loops and the β 1 C-terminus.

SBP is best modeled to fit this pocket in the β -furanose form, the most common form in solution. Positively charged histidine and arginine groups tie the phosphate groups in place at either end of the binding pocket. The hydrolyzed C1 phosphate is buried into the enzyme and forms hydrogen bonds and salt bridges with H13, H176, R12, and R69. In addition, the C1 phosphate coordinates to a Mg^{2+} ion which is not necessary for catalysis, but improves binding coordination. The C7 phosphate hydrogen bonds with H244 the second monomer, and interacts with H178 and R181 as well. Carbons 2 and 3 form part of the furanose ring and the hydroxyl groups on these carbons point towards the protein forming hydrogen bonds (Y102 and E99 to C2 and Y102 and T25 amide C3).

FBP coordinates to Shb17 in much the same fashion, indicating that the mechanism for hydrolysis is probably the same. SBP forms two more hydrogen bonds to shb17 than FBP between C3-OH and Y102 and the C1-P and R12. The formation of the furanose ring in SBP, which is thermodynamically more favorable than the linear form, makes the distance between phosphates similar (10.7 and 10.6 Å). The extra hydrogen bonds in

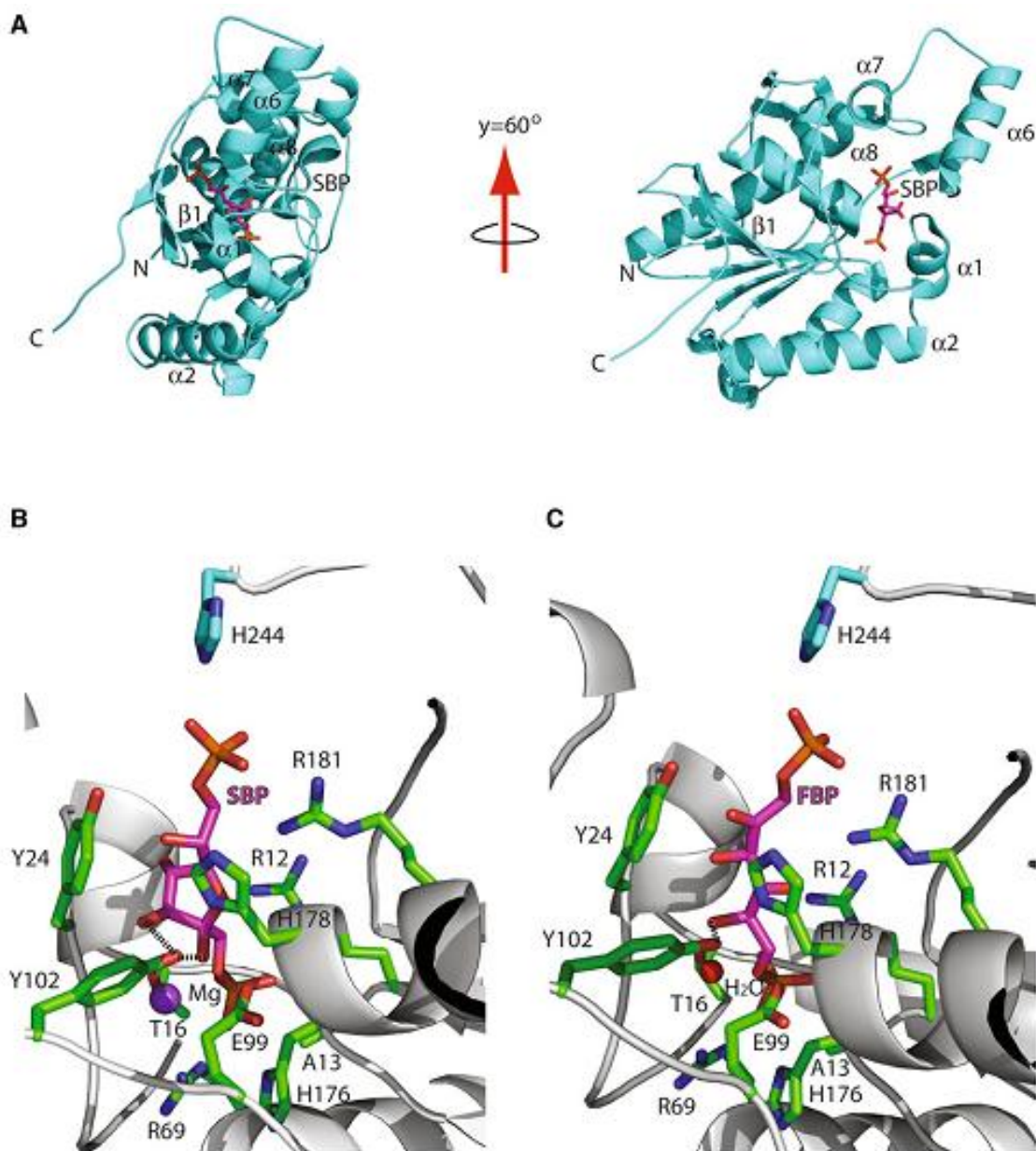


Figure 3.5. The structure of Shb17. A) Overall structure of Shb17 in complex with SBP at two angles. B) A more detailed view of SBP in the active site, residues shown to interact with SBP are shown in stick form. C) The active site of Shb17 with FBP bound.

conjunction with the preferred sugar conformation probably leads to the higher affinity of Shb17 observed for SBP over FBP.

3.6 Fructose bisphosphate aldolase makes SBP *in vivo*

There are two possible routes to synthesize SBP: Either S1P could be phosphorylated, or SBP could be synthesized by aldol addition of DHAP to E4P (Figure 3.6). S1P synthesis would be consistent with accumulation of S1P observed in the mutant. Similarly OBP could be synthesized from O1P or from DHAP and a pentose-5-P. To better understand the synthesis of these molecules, ($^{13}\text{C}_6$)glucose:unlabeled glucose was fed to wild type yeast in a 70:30 ratio. This method results in the partial labeling of the nonoxidative PPP intermediates. The most common labeled forms of SBP were $^{13}\text{C}_3$ -SBP and $^{13}\text{C}_4$ -SBP, which are consistent with synthesis directly from a triose and a tetrose by aldolase condensation. S7P labeling echoed the SBP pattern. Similarly the majority of OBP and O8P contained 3 or 5 ^{13}C carbons (Figure 3.7). The signal-to-noise ratio was too low to measure partially labeled S1P and O1P.

The role of fructose bisphosphate aldolase was further explored by kinetic flux profiling. Because fructose bisphosphate aldolase is an essential gene, the gene was knocked down by decreased abundance mRNA perturbation (DAmP)⁷⁶ rather than deleted. Cultures were established for each of four strains (wt, *shb17Δ*, FBA1-DAmP, and a *shb17Δ*/FBA1-DAmP double mutant) on unlabeled media, then switched to ($^{13}\text{C}_6$)glucose labeled media. The metabolism of these samples was quenched, and metabolites extracted at regular intervals, enabling us to monitor incorporation of ^{13}C into metabolite pools as carbon flows from glucose into the PPP.

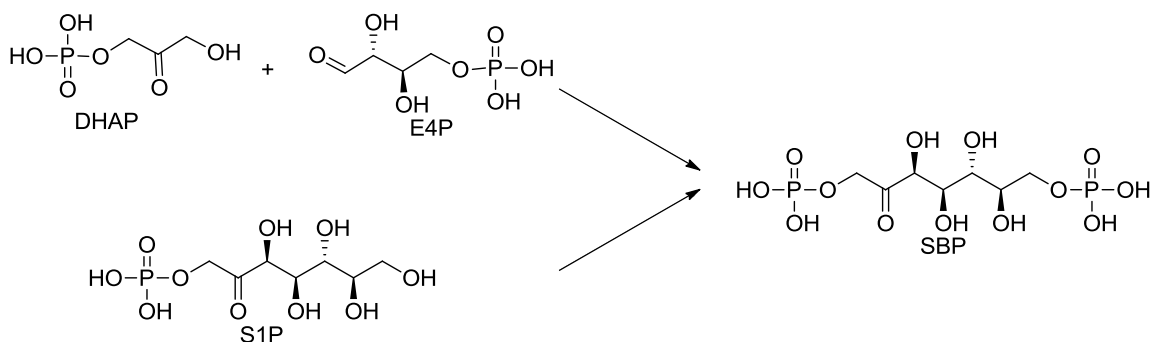


Figure 3.6. Potential biosynthetic routes to SBP.

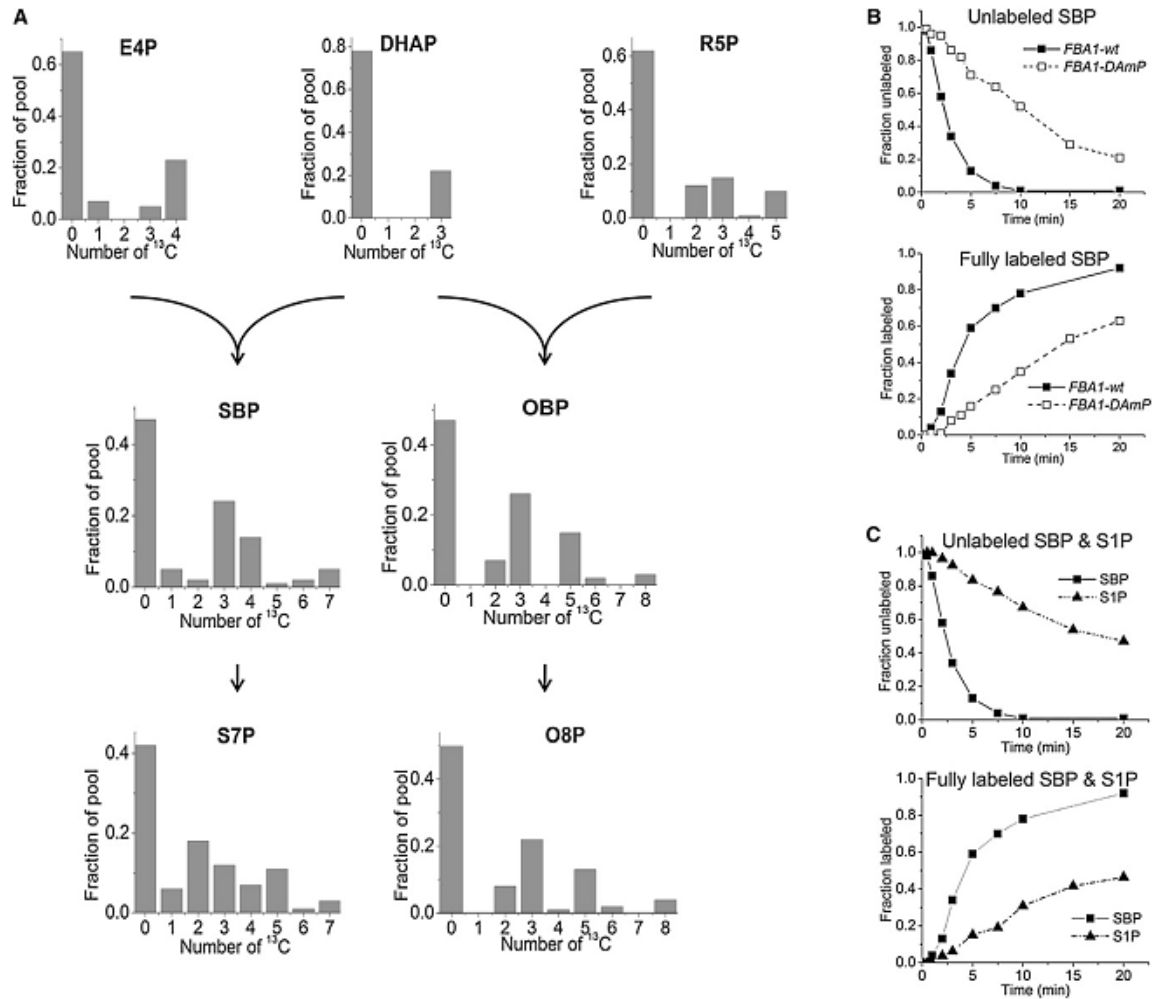


Figure 3.7. SBP and OBP are synthesized *in vivo* by fructose bisphosphate aldolase. A) Labeling patterns of substrates and products of Shb17 after *shb17Δ* cells were switched to 70:30 unlabeled glucose:($^{13}\text{C}_6$)glucose. B) SBP labeling kinetics of *shb17Δ* and *shb17Δ*/FBA1-DAmP strains after a switch to ($^{13}\text{C}_6$)glucose. C) Kinetics of SBP and S1P labeling after *shb17Δ* was switched to ($^{13}\text{C}_6$)glucose.

The FBA-1-DAmP allele did not significantly affect flux into metabolites DHAP and E4P, which are substrates of fructose biphosphate aldolase. However, labeling of SBP and OBP was substantially slowed. Synthesis of SBP and OBP by phosphorylation would require S1P and O1P to be labeled faster than SBP and OBP. In fact, the opposite was true, and S1P and O1P labeled even slower than SBP and OBP, indicating that these molecules are not the synthetic route to the bisphosphates, but rather the products of SBP or OBP hydrolysis. Thus, we can conclude that SBP and OBP are synthesized by fructose bisphosphate aldolase *in vivo*.

3.7 Quantitative flux through SHB17 into the nonoxidative PPP

There are two routes leading to S7P synthesis, either from SBP via Shb17 or through the canonical PPP. To ascertain which route contributes most to S7P synthesis, cultures were grown on yeast nitrogen base and then switched to otherwise identical media containing (6-¹³C₁)glucose for 90 min. This incubation time allowed straightforward labeling reactions to reach a pseudo-steady state, but eliminated the majority of effects from scrambling reactions. The S7P product of the canonical PPP should be singly labeled at C7. If the Shb17 enzyme is used for S7P synthesis the product should be doubly labeled at carbons 1 and 7 (Figure 3.8).

If (6-¹³C₁)glucose is processed by the oxidative PPP, the product is (5-¹³C₁)ribulose-5-phosphate (Ru5P). Two units of Ru5P can then be epimerized and isomerized to (5-¹³C₁)xylulose-5-phosphate and (5-¹³C₁)ribose-5-phosphate respectively. Their reaction with transketolase would then produce (7-¹³C₁)S7P and (3-¹³C₁)GAP. Likewise processing of [6-¹³C₁]-glucose through glycolysis would result in (6-¹³C₁)F6P and (3-¹³C₁)GAP. Transketolase can synthesize (4-¹³C₁)E4P and (5-¹³C₁)Xu5P from these substrates. Using this (4-¹³C₁)E4P and another unit of (6-¹³C₁)F6P transaldolase again makes (6-¹³C₁)glucose and (3-¹³C₁)GAP. Therefore, canonical PPP processing of (6-¹³C₁)glucose leads to (7-¹³C₁)S7P from either flux direction.

On the other hand, fructose bisphosphate aldolase can convert a unit of (3-¹³C₁)DHAP and (4-¹³C₁)E4P to (1,7-¹³C₂)SBP. When Shb17 hydrolyzes the SBP, (1,7-¹³C₂)S7P is formed. The role of aldolase in SBP synthesis is therefore central to the feasibility of this strategy.

To validate this approach *shb17Δ* and wild type cultures were compared. Less than 1% of the S7P was labeled in the mutant strain. In the wild type strain, 5% of the S7P was doubly labeled, therefore we could conclude that the *shb17Δ* mutation shut down that synthetic route. The amount of doubly labeled SBP in the wild type was 25%, indicating that 20% of the S7P pool was the result of SBP hydrolysis. On a side note, 10% of the FBP was doubly labeled, but no doubly labeled glucose-6-phosphate was observed. FBP hydrolysis is therefore not a significant source of hexose-phosphates.

A similar approach was taken for other biosynthetic routes to S7P. The genes glucose 6-phosphate dehydrogenase *zwf1*, transaldolases *tal1* and *nqm1*, and transketolases *tkl1* and *tkl2* were also targeted for deletion. Obstruction of the oxidative PPP by *zwf1* mutation resulted in an increase in flux to 35% S7P synthesized via Shb17. Removal of transaldolase activity by deletion of *tal1* and *nqm1* increased flux through Shb17 to 90%. A triple mutant however only had a flux of 55%. This strain had a growth defect that could explain this difference by overall disregulation of fluxes. Transketolase is necessary for the formation of E4P the direct precursor to SBP, and a double mutant of both *tkl1* and *tkl2* also showed no flux through Shb17.

These findings are consistent with three routes to R5P production through the oxidative PPP, the nonoxidative PPP, and Shb17. However a *zwfΔtal1Δshb17Δ* triple mutant was not lethal, although growth was stunted (Figure 3.8 panel D). This is explained by transketolase ability to make Xu5P and E4P from the glycolysis intermediates GAP and F6P on its own. Xu5P can be converted in two steps to R5P via non-effected enzymes, however this route also produces E4P stoichiometrically which cannot be used without aldolase activity. Thus solo transketolase production of R5P is a wasteful endeavor for the cell. Both the transaldolase and *shb17* routes require transketolase activity and therefore a *zwfΔtkl1Δtkl2Δ* triple mutant was lethal.

Further experiments explored the regulation of ribose production through Shb17. We reasoned that fast growth on YNB was not necessarily the most important condition requiring utilization of the Shb17 enzyme. Shb17 provides a thermodynamically driven route to S7P, and therefore may pull glycolysis intermediates toward synthesis of ribose when NADPH production is not needed. If this hypothesis was true, eliminating the need for NADPH production should increase flux through Shb17. The primary uses of NADPH are amino acid, nucleic acid base, fatty acid and sterol biosynthesis. Therefore, representatives of each of these categories and combinations thereof were added to the

media, and flux through wild type yeast was measured. Flux through Shb17 increased from 20% to almost 50% with all additives. Together this data supports the idea that Shb17 provides a thermodynamic driving force for riboneogenesis.

3.8 *SBP17 oscillates with ribosomal transcripts in yeast metabolic cycle*

Next the gene expression patterns⁷⁷ were analyzed for further evidence of a riboneogenic role for *shb17*. Yeast can synchronize gene expression under poor nutrient conditions so that the culture oscillates between fermentation and respiration. Expression of *shb17* occurs concurrently with ribosomal proteins Rpl17B, Rpl6B, and Rps28B and just prior to the DNA replication cluster containing proteins Rnr1, Pol12 and Cdc45 (Figure 3.9). The timing indicates that Shb17 is expressed at the peak demand for RBP where it is used for the assembly of ribosomes.

Shb17 aids the production of R5P from glycolysis intermediates in concert with four other enzymes transketolase Tkl1, fructose biphosphate aldolase Fba1, ribulose-5-phosphate epimerase Rpe1, and ribose-5-phosphate ketol-isomerase Rki1. Together they can convert 1 mol F6P and 3 mol GAP to 3 mol of R5P, and these reactions make up a previously undescribed pathway of riboneogenesis. Shb17 is expressed in parallel with Tkl1 and Rki1, while Rpe1 is not expressed temporally, giving credence to the coordination of these enzymes for a particular metabolic function.

The oxidative PPP enzyme Zwf1 is anticorrelated with *shb17* production, which would support the hypothesis that the cells utilize the riboneogenesis pathway when NADPH is not in demand. Interestingly, Tal1 is also expressed at opposite time intervals to Shb17. In the canonical nonoxidative PPP, transketolase and transaldolase work together to produce 3 mol of R5P from 2 mol F6P and 1 mol GAP. However, because Tal1 is expressed at a different metabolic phase than Tkl1, Shb17, and Rki1, Tal1 may not work in coordination with Tkl1 to produce R5P. Rather we presume that riboneogenesis is conducted by Tkl1, Shb17, and Rki1.

3.9 Conclusions

Here we have characterized the enzyme Shb17, previously known as YKR043c of unknown function. Shb17 hydrolyzes SBP to S7P, completing a thermodynamically driven step that drives riboneogenesis from glycolytic trioses. Other component enzymes

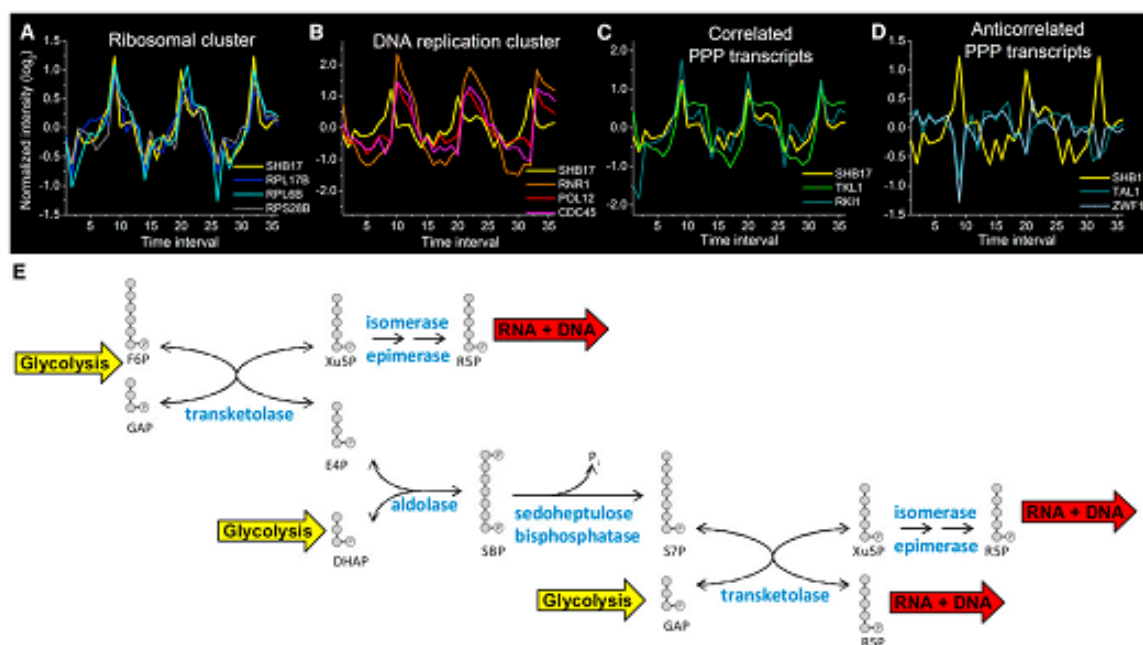


Figure 3.9. Shb17 is expressed in concert with the yeast metabolic cycle. Gene expression was measured every 25 min over three yeast metabolic cycles⁷⁷ for A) ribosomal transcripts, B) DNA replication genes, and C-D) PPP enzymes. E) Proposed riboneogenesis pathway, supported by correlation and anticorrelation of *shb17* expression and enzymes of the PPP.

of the newly described pathway include transketolase Tkl1, ribulose-5-phosphate epimerase Rpe1, and ribose 5-phosphate isomerase Rki1. Riboneogenesis is regulated by biosynthetic (ribose 5-phosphate) and redox (NADPH) demands on the cell, which do not always occur in the same phase of the yeast metabolic cycle. These findings can explain an increase in S7P concentration during inhibition of the oxidative PPP that previous models could not.⁷⁸

Recent crystallography studies of Shb17 demonstrated FBP could complex with the enzyme.⁷⁵ However Shb17 deletion did not cause accumulation of FBP, nor was [1,6-¹³C₂]-hexose phosphate observed in flux studies. Kinetic studies were able to demonstrate a higher affinity of Shb17 for SBP than FBP, and the crystallographic structure explains this difference. SBP coordinates to Shb17 in the more stable β -furanose form, unlike the linear form of FBP, and makes two additional hydrogen bonds

to Shb17. This study demonstrates the power of combining metabolomics and structural biology techniques to discern the function of an enzyme and its endogenous substrate.

While SBP and OBP are not reported in most of the metabolic pathways of both microbial and animal centric databases, they are present and abundant in multiple species. Flux experiments in the distantly related fission yeast *S. pombe* also show formation of doubly labeled S7P. In *E. coli*, transaldolase mutants fed xylose run the nonoxidative PPP to S7P where it builds to high enough concentrations to be phosphorylated to SBP by phosphofructokinase. It can then be cleaved to DHAP and E4P. Seven- and eight-carbon sugars also appear to be important in mammals. Sedoheptulose kinase must be down regulated for macrophage activation, and SBP levels are increased in tumor material and oncogene transformed mouse cell lines.^{70,79}

An alternative form of the PPP, termed the L type pathway was reported in the mammalian liver.⁶⁷ Although this pathway involves formation of SBP from DHAP and E4P, there are several important differences. The pathway relies on an uncharacterized phosphotransfer reaction between OBP and S7P, not a sedoheptulose bisphosphatase. The phosphotransfer reaction is not thermodynamically driven, and therefore the pathway lacks a driving force necessary for determining flux in a particular direction. In addition, the steps of the L type pathway result in the same products as the canonical pathway. In contrast, Shb17 cleaves a high-energy phosphate bond and drives the pathway in the direction of ribose phosphate synthesis.

Riboneogenesis and the Calvin cycle are closely related. In these light-independent reactions of photosynthesis, SBP is formed from an aldolase catalyzed DHAP and E4P condensation. SBP is then enzymatically hydrolyzed to S7P, and a unit of GAP is consumed to produce R5P and Xu5P. These are isomerized and phosphorylated to form ribulose-1,5-bisphosphate which condenses with carbon dioxide in the carbon fixation step. There are important differences between these pathways as well. The bisphosphatase enzymes of the Calvin cycle are members of the phosphoglycerate mutase family and are only very distantly related to the enzymes of riboneogenesis, including Shb17. These enzymes are regulated by light intensity and are located only in chloroplast stroma. Shb17, on the other hand, is found in the cytoplasm and is localized there to help regulate glycolysis and the PPP.

Demand for biosynthetic intermediates and redox carriers is constantly changing in response to growth rate, growth cycle, oxidative stress and nutrient availability.

Riboneogenesis empowers the cell to maintain homeostasis in the face of these fluctuating demands on the products of the pentose phosphate pathway.

3.10 Methods

Chemicals and Enzymes. HPLC-grade water, methanol and acetonitrile (OmniSolv, EMD Chemical), fructose-6-phosphate disodium salt and erythrose (70% w/v syrup) were obtained from VWR International (West Chester, PA). Tributylamine, acetic acid, magnesium chloride (98%), ribose-5-phosphate barium salt hexahydrate (R99%), thiamine-pyrophosphate, spinach aldolase (0.27 U/mg), rabbit muscle aldolase (11.5 U/mg), transketolase (0.16 U/mg), and triose phosphate isomerase (4120 U/mg) were purchased from Sigma-Aldrich (St. Louis, MO). Fructose-1,6-bisphosphate trisodium salt octahydrate (98%) was obtained from AK Scientific, Mountain View, CA. Zinc acetate dehydrate (99.7%) was from J.T. Baker, Phillipsburg, NJ, and tris(hydroxymethyl)methanamine (99+%) was from Thermo Fisher Scientific, Pittsburg, PA.

Yeast Strains and Media Components. Strains were derived from the synthetic genetic analysis (SGA) deletion set (BY4743 background)⁸⁰ with the *genotype MATa ura3Δ0 leu2Δ0 his3Δ1 lys2Δ0 met15Δ0 can1Δ::LEU2⁺-MFA1pr-HIS3/CAN1 ykr043Δ::kanMX* and a *yar047cΔ::kanMX* control in the same background. Prototrophic deletions were also created by homologous recombination using the YKR043CΔ::ClonNAT allele amplified by PCR from the above deletion. Prototrophic controls were wild-type FY4 (Mat a) and FY5 (Mat α).⁸¹ Ergosterol, palmitate, amino acids, and all other media components were obtained from Sigma-Aldrich (St. Louis, MO). Polysorbate 80, FCC was obtained from Spectrum Chemicals (New Brunswick, NJ). Ultra fatty acid free BSA was obtained from Roche (Indianapolis, IN). [¹⁵N]-NH₄Cl, (¹³C₆)glucose and (6-¹³C₁)glucose were obtained from Cambridge Isotope Laboratories (Andover, MA). Minimal media was made with 6.7 g/l Difco Yeast Nitrogen Base without amino acids plus 2% (w/v) glucose. For the SGA-derived auxotrophic strains, minimal media were supplemented with 20 mg/l uracil, 20 mg/l L-methionine, and 30 mg/l L-lysine HCl. Plates were prepared with 15 g/l triple washed ultrapure agarose.⁸²

For Shb17 flux experiments, amino acid, adenine sulfate and uracil supplements were added to concentrations recommended in Methods in Yeast Genetics.⁸³ For the amino acid supplementation, alanine, cysteine and proline, which are not included in the supplementation recipe in Methods in Yeast Genetics, were added at 35 mg/l, thereby providing supplementation for 17 amino acids (Katja Schwartz, personal communication). Ergosterol stock was 2 µg/ml in 50/50 (v/v) Tween 80 / ethanol, dissolved by overnight stirring, then added to yield a final supplementation of 20 mg/ml. Sodium palmitate was prepared at a concentration of 1 mM in 150 mM NaCl solution at 70 °C. The sodium palmitate was complexed at 37 °C to 0.2 mM fatty-acid free BSA in 150 mM NaCl as described by Seahorse Biosciences; final mixture was 40 mM palmitate with 6.8 µM BSA. Fatty acid-BSA complexes were stored up to 1 week at – 20 °C. Table S4 provides full recipes for all media. For experiments involving short term ¹³C-labeling (kinetic flux profiling of the DaMP aldolase mutants or the (6-¹³C₁)glucose experiments) and absolute quantitation of FBP and SBP,²⁵ yeast were grown on filters on an agarose-media support as described.^{52,84} This allowed rapid, non-disruptive transfer of cells from unlabeled to labeled media, and rapid quenching of metabolism. All data was corrected for naturally occurring ¹³C.⁵²

Screening for Metabolic Phenotypes. Single colonies of yeast deletion and control strains (n = 4 of each) were grown overnight to saturation at 30 °C in minimal medium. Cultures were set back to OD₆₀₀ 0.1 in 25 ml media and allowed to grow at 30 °C to mid-log phase (OD₆₀₀ between 0.4 to 0.6). Cells were harvested by vacuum filtering the cultures onto 47 mm diameter 0.45 µm nylon filters. Metabolism was quenched by quickly placing the filter cell side down in 0.8 ml of -20 °C extraction solvent (40/40/20 acetonitrile/methanol/water). The cells were allowed to sit in the extraction solvent at -20 °C for 15 min, at which time cells were washed off of the filter with an additional 200 µl solvent. The solvent-cell mixture was then centrifuged. The supernatant was removed and placed on ice. Pellets were resuspended in 200 µl fresh solvent, and a second extraction was performed for 15 min at 5 °C. The resulting mixture was then centrifuged, and the two supernatants were combined. Aliquots were dried under N₂ (g) and resuspended in HPLC-grade water prior to LC-MS analysis.

LC/MS Analysis. Instrumentation was an Accela U-HPLC system, HTC PAL autosampler, and a stand-alone orbitrap mass spectrometer with an electrospray ionization source (Thermo Fischer Scientific). The reverse-phase ion pairing LC method consists of a Phenomenex Synergy Hydro-RP column (100 x 2 mm, 2.5 μ m particle size, Phenomenex, Torrance, CA) and an aqueous/organic gradient run at 200 μ l/min. Mobile phase A was 97:3 water:methanol with 10 mM tributylamine and 15 mM acetic acid. Mobile phase B was methanol. The gradient was 0 min, 0% B; 2.5 min, 0% B; 5 min, 20% B; 7.5 min, 20% B; 13 min, 55% B; 15.5 min, 95% B; 18.5 min, 95% B; 19 min, 0% B; 25 min, 0% B. The column temperature was maintained at 25 $^{\circ}$ C, and the autosampler at 5 $^{\circ}$ C. The injection volume was 10 μ L. The stand-alone orbitrap mass spectrometer was run in negative ionization mode. Data was collected at 100,000 resolution at 1 Hz under automatic gain control (target 3×10^6 ions) and a maximum injection time of 100 μ s. Instrument run parameters were sheath gas flow rate at 25 (arbitrary units), auxiliary gas flow rate at 8 (arbitrary units), sweep gas flow rate at 3 (arbitrary units), spray voltage at 3 kV, capillary temperature at 325 $^{\circ}$ C, capillary voltage at -50 V, tube lens voltage at -100 V. The scan range was 85 to 1000 m/z , with a more restricted scan range at certain points in the run to avoid artifacts from phosphate and sulfate accumulation in the orbitrap.³² Peaks differing between wild-type and knock-out strains were determined using in-house developed, open-source software MAVEN,⁸⁵ whose algorithms closely resembled those of XC-MS.⁴⁴ MAVEN was also used to quantitate isotope-labeled forms. To obtain MS/MS and MS/MS/MS fragmentation patterns, we used a similar chromatography method to the above³² coupled by negative mode electrospray ionization to either a triple quadrupole mass spectrometer or an ion trap-orbitrap hybrid mass spectrometer. The number of carbons and nitrogens in each molecule was determined by the method of Hegeman, et al.⁸⁶ Yeast cultures were grown with uniformly isotopically labeled nutrients for >20 generations to ensure complete labeling of metabolites prior to LC-MS analysis.

Synthesis of Sedoheptulose-1,7-Bisphosphate. An aqueous solution of cofactors, substrates, and buffers was made with the given final concentrations: thiamine-pyrophosphate (1.1 μ M), fructose-6-phosphate (2.5 mM), fructose-1,6-bisphosphate (5.7 mM), magnesium chloride (110 μ M), and tris(hydroxymethyl)methanamine (4.7 mM). The enzymes rabbit muscle aldolase (750 μ l of 0.1 U/ μ l in 50 mM Tris), transketolase,

(50 μ l of 0.0032 U/ μ l in 50 mM Tris) and triose phosphate isomerase (78 μ l of 9.58 U/ μ l in 50mM Tris) were added to 6.6 ml of the cofactor and substrate solution to produce 7.5 ml of final reaction mixture containing thiamine-pyrophosphate (1 mM), fructose-6-phosphate (2.2 mM), fructose-1,6-bisphosphate (5 mM), magnesium chloride (100 mM), tris(hydroxymethyl)methanamine (10 mM), rabbit muscle aldolase (10 U/ml), transketolase (0.021 U/ml) and triose phosphate isomerase (100 U/ml). The reaction was incubated overnight at 37 °C and then flash frozen and stored at -80 °C until needed. The reaction was characterized by direct infusion negative mode ESI-MS. Based on these data, and assuming equal ionization efficiencies between phosphosugars, the reaction conversion was 27%.

Due to the formation of xylulose-5-phosphate as a side product of the transketolase reaction with fructose-6-phosphate and glyceraldehyde-3-phosphate, an octulose-bisphosphate was also formed. SBP purity was ~80% of the C7 and C8 sugar bisphosphates. To remove other byproducts, SBP was further purified by reversed phase ion pairing LC using a larger scale version of the method of Lu et al.³² Specifically, the column was Phenomenex AXIA Synergi 4 μ m Hydro-RP 80 Å, 250 x 21.2 mm. Mobile phase A was 97:3 water:methanol with 10 mM tributylamine and 15 mM acetic acid. Mobile phase B was methanol. Flow rate was 15 ml/min. The gradient was 0 min, 0% B; 5 min, 0% B; 10 min, 20% B; 20 min, 20% B; 35 min, 65% B; 38 min, 95% B; 42 min, 95% B; 43 min, 0% B; 50 min, 0% B. Injection volume ranged from 1.5 to 4 ml. 15 to 30 ml fractions were collected, tested for presence of SBP and FBP by LC/MS, and concentrated on a SpeedVac. This purification resulted in a final mixture containing FBP:SBP:OBP in the approximate ratio 2:1:0.25.

Synthesis of Sedoheptulose-1-Phosphate. An aqueous solution of cofactors, substrates, and buffers was made with the given final concentrations: fructose-1,6-bisphosphate (25.4 mM), erythrose (63.4 mM), zinc acetate (6.3 mM) and tris(hydroxymethyl)methanamine (58.5 mM). The enzymes spinach aldolase (28 μ l of 0.05 U/mL in 50mM Tris) and triose phosphate isomerase (1.5 μ l of 9.58 U/mL in 50mM Tris) were added to 110 μ l of the cofactor and substrate solution to produce 139 ml of final reaction mixture containing fructose-1,6-bisphosphate (20 mM), erythrose (50 mM), zinc acetate (5 mM) and tris(hydroxymethyl)methanamine (50 mM), aldolase (10 U/ml), and triose phosphate isomerase (100 U/ml). The reaction was incubated overnight at 37

°C and then flash frozen and stored at -80 °C until used. The reaction conversion was determined to be 54% by LC-MS/MS.

Synthesis of D-Glycero-D-Altro-Octulose-1,8-Bisphosphate. An aqueous solution of cofactors, substrates, and buffers was made with the given final concentrations: fructose-1,6-bisphosphate (25 mM), ribose-5-phosphate (62.5 mM), zinc acetate (5 mM) and tris(hydroxymethyl)methanamine (50.4 mM). The enzymes spinach aldolase (40 µl of 0.05 U/µl in 50 mM Tris) and triose phosphate isomerase (2.1 µl of 9.58 U/µl in 50mMTris) were added to 160 µl of the cofactor and substrate solution to produce 139 µl of final reaction mixture containing fructose-1,6-bisphosphate (20 mM), ribose-5-phosphate (50 mM), zinc acetate (5 mM) and tris(hydroxymethyl)methanamine (50 mM), aldolase (10 U/ml), and triose phosphate isomerase (100 U/ml). The reaction was incubated overnight at 37 °C and then flash frozen and stored at -80 °C until used. The reaction conversion was measured at 42% by LC-MS/MS.

Rapid LC-MS/MS Method Appropriate for Analyzing SBP in Low Complexity Samples. Samples were injected onto a Phenomenex Luna NH2 5 mm 100 Å 50 x 2.0 mm column at 15 °C, 10 µl injection volume. Mobile phase was a 12 min gradient of 20 mM NH₄HCO₃, 20 mM NH₄OH 95/5 H₂O/CH₃CN (A) and CH₃CN (B) flowing at 0.15 ml/min. From 0-6min B was decreased from 30% to 0, holding at 0 until 10 min, then back to 30% at 10.5 min. The LC was coupled to a Finnigan TSQ Quantum Ultra triple quadrupole MS (Thermo Electron Corporation, San Jose, CA) with an ESI source running in negative ionization mode. ESI spray voltage was 3000 V. Nitrogen was used as the sheath gas and auxiliary gas at 30 psi and 10 psi, respectively. Argon was the collision gas at 1.5 mTorr. Scan width was 0.1 *m/z* and scan time was 0.1 s. Capillary temperature was 325 °C. Monitored parent-product ions (collision energy) were 339-97 (25) FBP, and 369-97(25) for SBP.

Absolute Quantitation of FBP and SBP. Wild-type liquid cultures were started in YNB + 2% (¹³C₆)glucose, grown overnight at 30 °C, then set back to ~4 x10⁶ cells/ml as measured by Coulter counter. When cells reached ~1 x10⁷ cells/ml, 1.7 ml aliquots were filtered onto solid agarose plates with identical media supplementation. Once cultures reached mid-log phase (~2.5 x10⁷ cells/ml), filter cultures were transferred to pre-chilled

plates containing 0.8 ml of -20 °C 40/40/20 acetonitrile/methanol/water containing either 2.1×10^{-5} M FBP or 2.0×10^{-7} M SBP. The remainder of the metabolite extraction protocol is described in the Experimental Procedures section under “*Screening for Metabolic Phenotypes*.” Identically grown cultures were used for determining cell count and median cell size. Detailed procedure is reported in Bennett et al., 2008.⁸²

Protein Purification and Enzymatic Assays. For expression in *E. coli*, the coding sequence of Shb17 was amplified from the genome by PCR and cloned within the NdeI and XhoI sites of the pET-15 vector (EMD Biosciences), which provides an N-terminal 6x histidine tag. The sequence verified plasmid was transferred to Rosetta 2 (DE3) pLysS cells (EMD Biosciences). Protein expression was induced with IPTG. Cells were lysed by freeze-thaw in 50 mM sodium phosphate pH 7, 300 mM NaCl, 1 mM PMSF. Protein was bound to HisPur Cobalt resin (Thermo Scientific) and eluted in the same buffer with the addition of 150 mM imidazole. The purified enzyme was buffer exchanged into 10 mM Tris-HCl pH 7.0, 50 mM NaCl, 1 mM DTT using Econopac desalting columns (Biorad). Enzymatic activity was identified by incubating purified Shb17 protein with SBP, S1P and OBP at approximately 1000 ng/ml in a buffer of 10 mM Bis-Tris-Propane-HCl, 10 mM MgCl₂, 1 mM DTT pH 7.0. After 20 min of incubation at 30 °C, reactions were quenched by placing at 5 °C, adding acetonitrile to 20% final volume, and vortexing. Analysis was by LC-MS or targeted LC-MS/MS.

For kinetic experiments, all substrates were dissolved in water. C-terminally His₆-tagged Shb17 protein was quantified by Bradford assay (Biorad). Reactions contained 10 mM Bis-Tris-Propane-HCl pH 7.0, 10 mM MgCl₂, 1 mM DTT, 10 µg/ml BSA, and Shb17. Shb17 concentrations ranged from 2.5 to 8 mg/ml for reactions with FBP, and from 0.1 to 1.25 mg/ml for reactions with SBP. Note that the SBP solutions also contained residual FBP used during the SBP synthesis. Reactions in 30 µl volume were allowed to proceed for 15 min at 30 °C before quenching by the addition of 30 µl 20% acetic acid. Samples were diluted prior to LC/MS analysis. Calibration curves were prepared in parallel in an identical matrix lacking Shb17. A rapid LC-MS/MS method was developed and validated for the quantitation of SBP and FBP to accommodate the large number of samples generated during kinetic experiments.

Crystallography. Crystals of SHB17(H13A) were grown at room temperature by hanging-drop vapor diffusion using 2 μ l of protein (29 mg/ml) and 2 μ l of reservoir solution. Crystals were found using a reservoir solution of 0.1 M HEPES 7.5, 10% isopropanol, 22% PEG4K, 4% glycerol, with 0.03 mg/ml trypsin added to the protein solution.⁸⁷ These crystals were then soaked for 10 min in well solution plus 10 mM sedoheptulose-1,7-bisphosphate, and then cryoprotected using Paratone-N oil (Hampton Research) and flash-frozen in liquid nitrogen. The structure of SHB17(H13A) with bound sedoheptulose-1,7-bisphosphate was solved by molecular replacement from the protein coordinates of SHB17(H13A) solved with fructose-1,6-bisphosphate (PDB code 3LL4). Briefly, diffraction data were generated using a home-source Rigaku FRE Superbright generator equipped with osmic confocal mirrors, and the data was collected on a Rigaku Saturn A200 image plate (Rigaku Americas, TX). Diffraction data were integrated and scaled using HKL2000.⁸⁸ Four molecules of Ykr043C were found using the molecular replacement program PHASER.⁸⁹

Additional density was found in the active site in the same position as the sugar moiety in the fructose-1,6-bisphosphate complex, however the resulting sugar had a clear ring conformation rather than a linear one. The density was best fit by a beta-furanose conformation of sedoheptulose-1,7-bisphosphate, which was modeled using REEL from the PHENIX package.^{90,91} Following structure solution and ligand modeling, the model was improved with alternate cycles of manual building and automated and manual water-picking using COOT⁹² and restrained refinement against a maximum likelihood target with 5% of the reflections randomly excluded as a free test set using REFMAC5,⁹³ as implemented using the CCP4 package (Collaborative Computational Project, number 4), as well as rebuilding and subsequent refinement using PHENIX.⁹⁰ The final model contains two dimers of SHB17(H13A), each representing residues 3-263, and shows excellent stereochemistry overall with 99.6% of the residues in the most favored and additional allowed regions of the Ramachandran plot.⁹⁴

CHAPTER IV

CHARACTERIZING CARBON FLUX THROUGH THE MARINE PROTOTROPH PROCHLOROCOCCUS

4.1 Introduction

The world's oceans house about half of the planet's primary production,⁹⁵ which directly impacts the microbial food web and global environment.^{96,97} Marine prototrophs produce 45-50 billion metric tons of dissolved organic carbon (DOC) per year⁹⁸ from only sunlight and dissolved carbon dioxide or bicarbonate. This production is the input for the aquatic food web and distributed as dissolved organic matter (DOM) to heterotrophic bacteria.⁹⁹ The heterotrophs also contribute to the DOM pool, often facilitated by viral lysis. Both phototrophs and heterotrophs are themselves food sources for protists and higher organisms (Figure 4.1). It is becoming more apparent that individual interactions between organisms contribute to the regulation and distribution of the metabolism of the community.^{100,101} Thus, a better understanding of carbon fixation and cycling through the

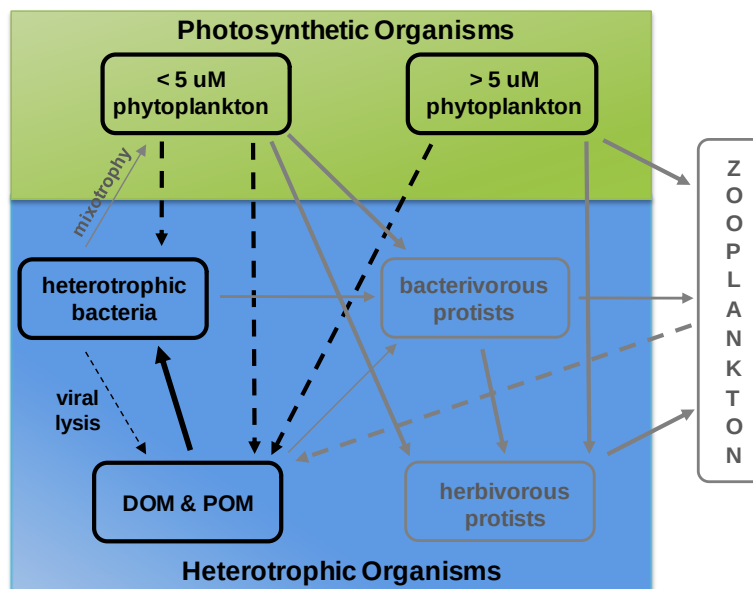


Figure 4.1. Marine microbial food web. Routes of consumption are marked with solid arrows and sources of nutrient release are marked with dashed arrows. Prokaryotic contributions are marked in black. Figure adapted from a review by Sherr and Sherr.^{99,102}

oceans via interactions of key species may contribute to a better understanding of the regulation of the global environment.

The genus *Prochlorococcus* represents the most abundant photosynthetic organisms on the planet¹⁰³ and fixes ~50% of all carbon in the ocean.¹⁰⁴ These cyanobacteria also produce ~60% of the oceanic chlorophyll¹⁰⁵ and regularly leak 25% of the carbon they fix during photosynthetic growth.¹⁰⁶ *Prochlorococci* are also the smallest oxygenic prototrophs both by cell size (0.6 μm in diameter) and genome length (1.64-2.68 Mbp).¹⁰⁷ In practice, the ocean behaves as buffer system for the planet. Changes in the temperature, pH, osmolarity, and oxidative environment of the ocean occur only occasionally during deep mixing events. *Prochlorococci* could therefore afford to streamline their genomes. Catalase activity, nitrogen fixation, and many regulatory features are not included in the genome. Hence, *Prochlorococci* benefit from a symbiotic relationship with a breadth of heterotrophs including γ -proteobacteria, α -proteobacteria, and yeast. When grown in the lab, these relationships provide protection in the form of peroxide removal and nitrogen sources.^{108,109} This symbiotic growth and abundance in ocean surface waters makes *Prochlorococcus* ideal for inclusion in model studies of the marine ecosystem.

The comparatively low numbers of regulatory features¹¹⁰ such as sigma factors, histidine kinases and response regulators, inspired the investigation of the mechanism of metabolic regulation in *Prochlorococcus* MED4.¹¹¹ Photosynthesis modulates over a the light and dark portions of a 24 h period (diel), and 2/3 of the carbon fixation in *Prochlorococcus* occurs between dawn and midday.^{112,113} Transcriptome studies found that these cyanobacteria accumulate proteins for maximum biomass generation before dawn,^{111,112,114} when they begin carbon fixation. As dusk approaches they express genes necessary for DNA replication and division before making a metabolic switch to glycogen storage and dividing once at night.¹¹¹ As mentioned previously, gene expression is not always indicative of metabolic state, and metabolomic studies would build upon these results to better characterize this metabolic regulation over the diel.

Herein we aim to build the technological foundation necessary to study the metabolism of marine microorganisms in coculture, and to observe the passage of nutrients between species. We believe that further understanding of the diel cycling,

interactions with other organisms and other ecological factors will contribute substantially to a robust multi-level model of marine microbial biology.

4.2 Technical Developments

There were important technical challenges to overcome before studying nutrient flow between symbiotic species would be possible. First, these are marine species found suspended in the surface waters of the open ocean. It is therefore highly biologically irrelevant to study these cultures on a supported filter, and there are currently no methods reported for fast switching liquid cultures from unlabeled media to labeled media. Secondly, we desired to study these organisms in coculture. While this is possible for genomics studies where the object of study is unique to an individual, metabolites are highly conserved and a physical barrier was therefore necessary. The solution reached was use of a dialysis membrane (MW <10 kDa), which allows the flow of nutrients between cultures, but keeps them physically separated. A dialysis membrane would also facilitate a speedy switch from one media to another by simply removing the bag from the outer media and placing it in the new environment. There will be some dilution of the new media with the old however, although this can be controlled by decreasing the inner:outer volume ratio. The rate of diffusion through the dialysis membrane will determine the rate of media exchange. Judging by the optimal time-scales of other dialysis membrane applications, the exchange will likely be slower than on filters. Untreated dialysis membranes were soon determined to be lethal to *Prochlorococcus* strain Vol1. The cellulose membranes of dialysis tubing coordinate metals, which are toxic to *Prochlorococci* because they have a reduced capacity to respond to oxidative stress. The same membranes were no longer toxic after they were soaked in a suspension of Chelex resin and then acid washed. Further control experiments were necessary before validating this approach for flux experiments.

4.3 Light/Dark Metabolic Phenotypes of *Prochlorococcus*

The small cell size, low cell densities and slow growth also posed problems for harvesting metabolites, as all of these limited the number of metabolites extracted. After optimization of the extraction procedure as described in Chapter 2, these methods were first validated by splitting a Vol1 culture grown in AMP media into two bottles, one clear

and one covered in black tape. These experiments compared identical cultures of Vol1 at experimental noon either grown as usual in light or in darkness for 1 h prior to sampling (Figure 4.2).

Experiments were completed in triplicate on each of two days. 70 metabolites were measured. Both positive and negative mode methods were run, however only negative mode was processed in full. Statistical analyses have not yet been performed on these data, however UDP-glucose (-2.0), UDPAG (-2.3), N-Acetyl-glucosamine (+1.4), 3-oxobutanoate (+60), palmitate (+1.2), phosphoenolpyruvate (-1.5), fumarate (-1.8), aspartate(-1.7), and asparagine (+139) stand out in the heat map. These metabolites may represent degradation of polysaccharide and lipid energy stores, decreased

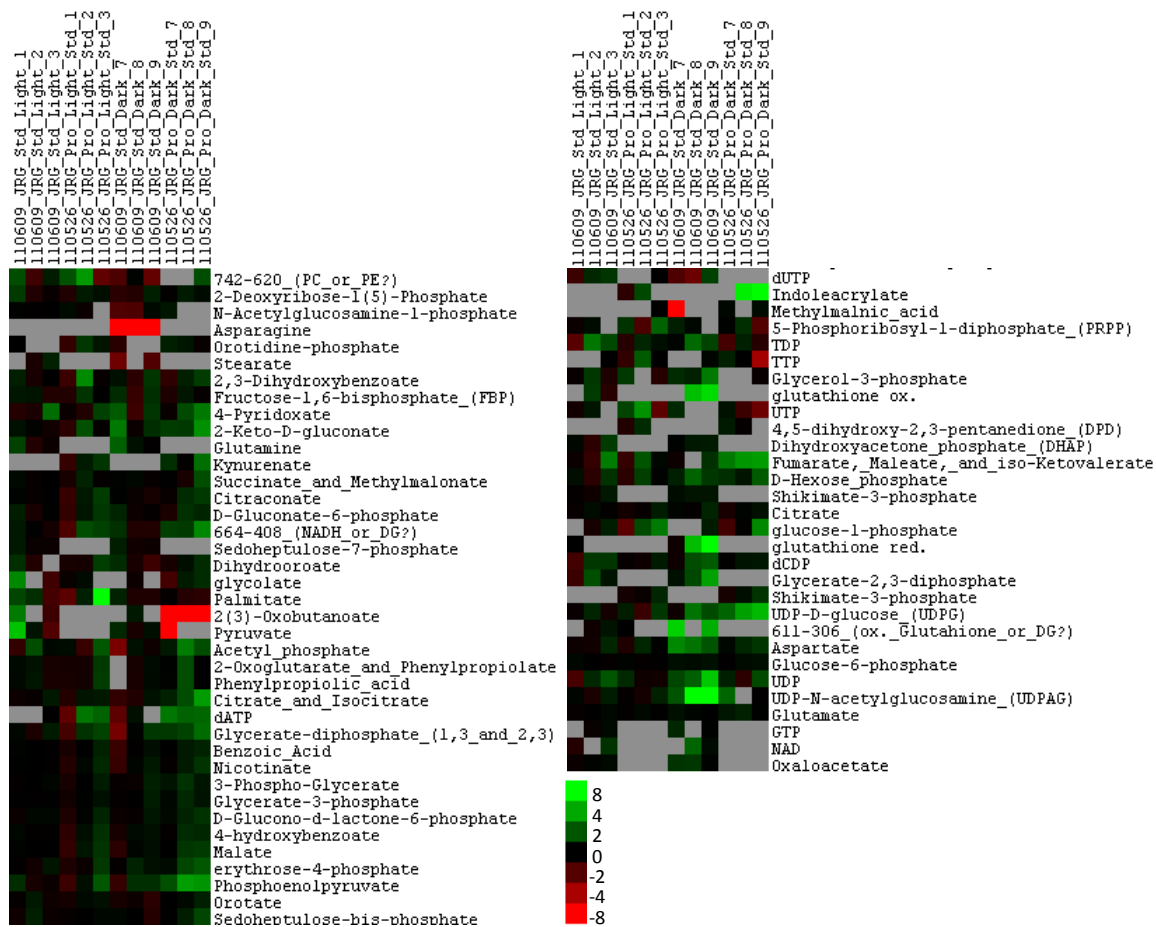


Figure 4.2. Heat map showing fold changes of triplicate light and dark samples one each of two days. Fold changes were calculated based on the average of the light samples for that day. Red denotes an increase, while green denotes a decrease compared to the light samples.

production of glycolysis intermediates as well as increased ammonia transport. These changes in metabolism are supported by the afore mentioned transcriptomics study.¹¹¹

4.4 Labeling *Prochlorococcus* Vol 1

Once we had validated the experimental design for reproducibility, statistical significance, and biological relevance, flux experiments were pursued. First the design of a targeted flux MRM method was necessary, including the choice of which SRMs and their corresponding labeled forms to include. There were two assumptions made in our design. First, labeled carbon is fixed by *Prochlorococcus*, and second, nutrients flow from *Prochlorococcus* Vol1 to *Alteromonas* EZ55. The set of metabolites was chosen based on ability to measure them, as evidenced by observations in previous experiments, as well as likelihood of importance, as indicated by the results the transcriptomics study. The metabolites of the Calvin cycle, pentose phosphate pathway, glycolysis, and glycogen synthesis were given particular consideration. The labeled forms of the SRMs were expanded from this list using the following criteria. The fully ¹³C labeled form of all metabolites was included. Singly labeled SRMs were added for metabolites with the fewest number of metabolic reactions separating the carbon fixation reaction and the synthesis of the metabolite, such as those in the Calvin cycle, pentose phosphate pathway, glycolysis and TCA cycle. The method also included SRMs that accounted for fully labeled major synthetic units for the majority of metabolites. For example, a two carbon labeled SRM was added for the acetyl unit of acetyl-CoA. No experiments using our methods had been performed previously on photosynthetic organisms, so two metabolites important to the Calvin cycle, sedoheptulose-1,7-bisphosphate and ribulose-1,5-bisphosphate, were added to the library by characterizing the fragmentation of standard solutions.

We desired to observe nutrient flow in two directions (Figure 4.3). Vol1 is expected to fix carbon and then pass some of these new nutrients to EZ55. For these experiments, ¹³C-bicarbonate was chosen as the labeled source of carbon. *Prochlorococcus* can fix this inorganic carbon, but it is not nutritionally accessible to the heterotroph *Alteromonas*. It is also known that EZ55 provides some form of support to Vol1 when grown in the laboratory,^{108,115} and it is possible that small molecules are responsible for this assistance. In this case, (2-¹³C)acetate was chosen as the carbon source. In initial

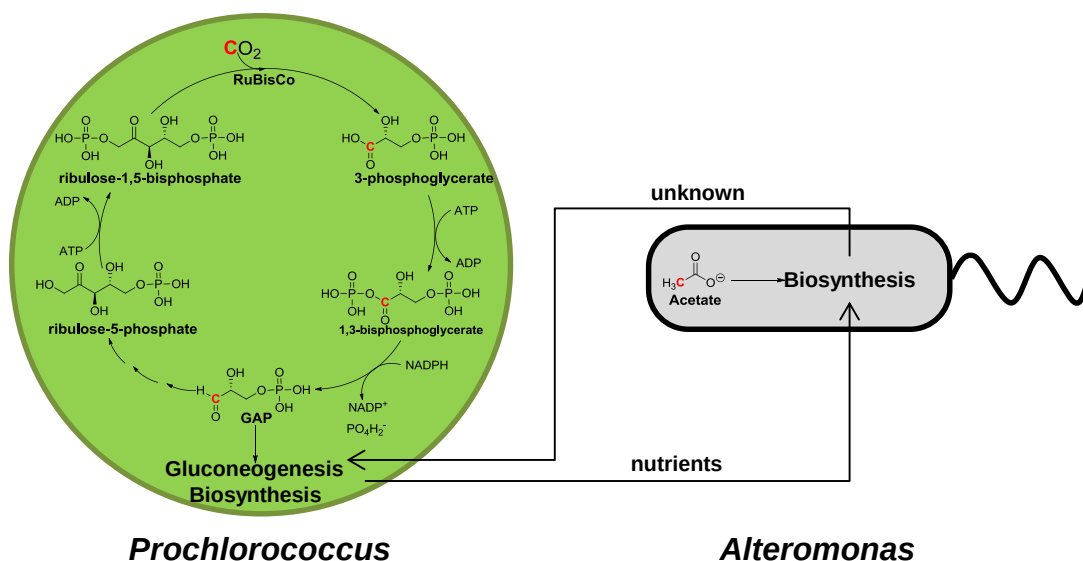


Figure 4.3 Flux between *Prochlorococcus* and a protective species.

control experiments where Vol1 was grown in the presence of acetate and Ez55 was grown in the presence of bicarbonate, no labeling was observed.

Experiments studying the Vol1 to EZ55 flux were attempted first because the nature of this relationship had been better characterized. Early attempts to label Vol1 were at first unsuccessful and several variables were optimized for better labeling. Vol1 was first given more time to acclimatize to higher bicarbonate concentrations, after which the sodium bicarbonate concentration could be increased to a maximum of 12 mM. Culture vessels were also sealed with parafilm, and the headspace of some cultures were limited to prevent exchange of atmospheric carbon dioxide. Using these simple designs to limit atmospheric carbon dioxide exchange, no improvement in labeling levels was observed. We also varied the length of time allowed for labeling and the number of passages into fresh labeled media. When the time was increased we observed an increase in the extent of labeling. During this optimization process, an elemental analyzer tandem continuous flow isotope ratio mass spectrometer was utilized to test the overall extent of labeling of the culture. This experiment indicated extensive labeling throughout the cell, however the upper limit of the linear range on this instrument is 8% labeling. Therefore we were unable to quantify this result. Efforts were also made to characterize potential issues with the LC-MS/MS detection method. Detection problems due to partial labeling and high salt content were expected. Of the six metabolites

checked, two demonstrated a higher signal intensity for adduct SRMs, a gluconate sulfate adduct and a palmitate chloride adduct. Little or no improvement was observed after thorough cleaning of the ion source.

4.5 Flux Experiments

Recently, a full flux experiment was attempted. For these experiments, a culture of *Prochlorococcus* Vol1 was split and grown in AMP media with and without ^{13}C labeled bicarbonate for at least 7 days. To three large sterile crystallization dishes with lids was added 250 mL AMP media containing ^{13}C -bicarbonate, and to another three was added 250 mL of the labeled Vol1 culture diluted into fresh AMP media. EZ55 cultures (~10 mL) were prepared in 12 dialysis bags and two were placed into each of the six dishes (Figure 4.4). The dishes were incubated in a sun box with aeration. Time zero samples were immediately sampled in triplicate by filtration from the remaining culture stocks. A *Prochlorococcus* Vol1 culture with natural abundance carbons was also sampled at this time. At 4 h, the dishes were removed from the sun box and one of the EZ55 dialysis bags from each dish was extracted. The dishes were returned until the 8 h time point was reached, when the remaining six EZ55 cultures were extracted for a total of 21 samples.

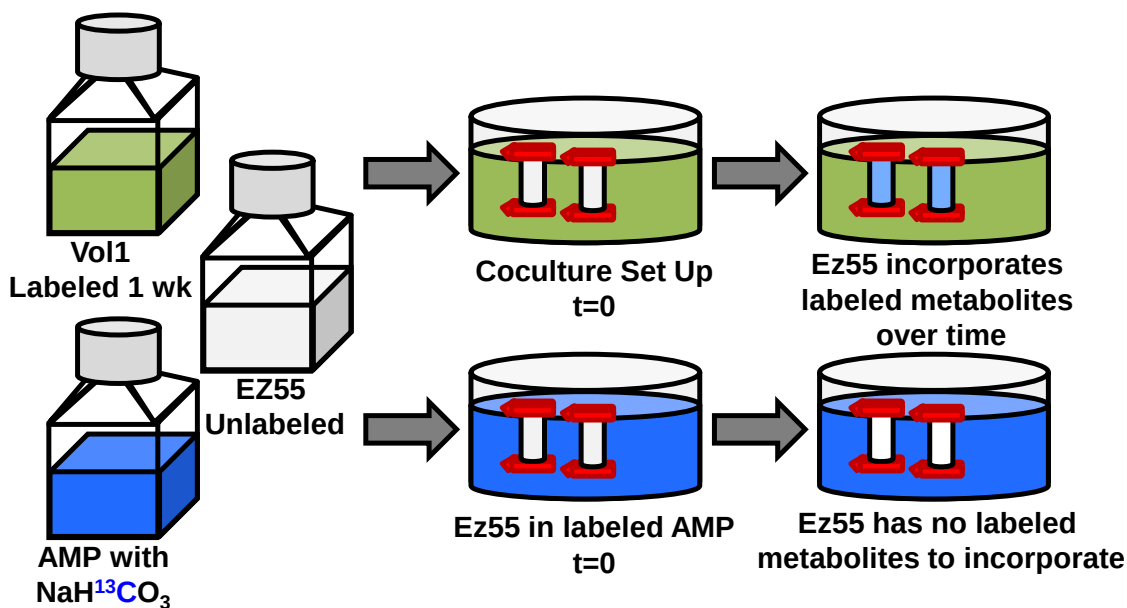


Figure 4.4 Experimental set up for coculture flux experiment.

Preliminary processing of this data set showed that the large glutamate pool was partially labeled up to 5.5% and fully labeled to 1.3 % (Figure 4.5). No fully labeled glutamate was observed for the ^{13}C -bicarbonate control.

4.6 Conclusions

We have developed a method for the extraction *Prochlorococcus* cultures. There is still much room for improvement; however, we have been able to collect relative concentration data for cultures grown in light and dark that appears to support earlier gene expression work. *Prochlorococcus* cultures are challenging to handle, and we have especially struggled to measure the extent of ^{13}C labeling in this species. Nonetheless, Vol1 mediated labeling of EZ55 glutamate pools has been measured. To our knowledge this is the first example of quantified nutrient flux between species in liquid coculture. We believe this liquid coculture method will be of importance to symbiotic phenomenon in many systems outside the scope of our current study.

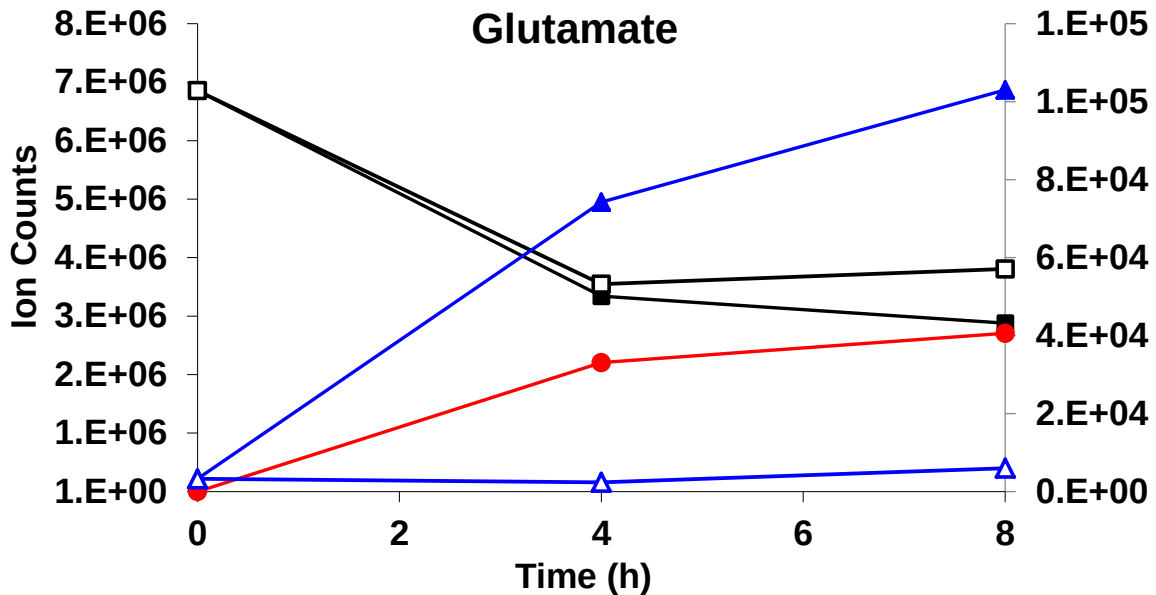


Figure 4.3. Integrated ion counts of time for $^{12}\text{C}_6$ -glutamate (■), $^{13}\text{C}_3$ -glutamate (▲), and $^{13}\text{C}_5$ -glutamate (●) in EZ55 cultures grown in the presence of ^{13}C -bicarbonate AMP (open) or ^{13}C labeled Vol1 (filled).

4.7 Methods

Strains and growth conditions. *Prochlorococcus* strain Vol1, is a derivative of parent strain MIT9215 which was isolated from the Sargasso Sea. *Alteromonas* strain EZ55 was isolated with MIT9215 as a culture contaminant. Each were maintained in the defined minimal medium AMP which consists of 28.1 g NaCl, 6.9 g MgSO₄, 5.49 g MgCl₂ x 7H₂O, 0.67 g KCl, 1.47 g CaCl₂ x 2H₂O per liter and solutions of 50 µM NaH₂PO₄, 0.8 mM NH₄Cl, and 6 mM NaHCO₃, and trace metals are added after autoclaving. The strains were incubated in a sunbox that mimics the gradient of natural light over the course of 24 h.

Dialysis membrane preparation. Dialysis membranes (Pierce Snakeskin #68100, 10,000 MWCO) were soaked in 5% Chelex for 48 h with orbital shaking. Then the membranes were rinsed six times in MilliQ quality water, and soaked overnight in 1N HCl. The membranes were rinsed another six times with MilliQ water, and soaked in MilliQ water until the day of experiment. The membranes were then tyndlized by bringing them to a boil three times in a standard microwave.

Determination of light/dark metabolic phenotypes. At around noon the Vol1 culture (3.5 x10⁸ cells/ml) was separated into two different batches, one was placed in a clear polycarbonate bottle (light) and the other into a taped polycarbonate bottle (dark). After one h, an aliquot (15 mL) was collected on a nylon filter and immediately transferred cell-side down to a 60 x 15 mm petri dish at -20 °C containing 1.3 mL of an extraction solvent (0.1 M formic acid in 1:2:2 water:methanol:acetonitrile) and internal standards (42.5 µM tris(hydroxymethyl)aminomethane and 17 µM benzoic acid). The samples were then allowed to extract for 20 min at -20 °C. The filters were then washed by pipetting the extraction solution over the surface for approximately 30 s. The solution was then centrifuged (5 min, 13.2 rpm, 16.1 rcf, 4°C) and 300 µL of the supernatants was added to each of two autosampler vials containing 26 µL of 15% ammonium bicarbonate. One vial was placed directly in the autosampler for analysis, and the other was frozen at -80°C for analysis in the opposite mode the following day. Dark samples are collected under red light. Samples are technical replicates. Experiments were completed in triplicate on each of two days.

Carbon flux from Vol1 to Ez55. For these experiments, a culture of *Prochlorococcus* Vol1 was split and grown in AMP media with and without ^{13}C -sodium bicarbonate (12 mM) for at least 7 days. To three large sterile crystallization dishes with lids was added 250 mL AMP media containing ^{13}C -bicarbonate, and to another three was added 250 mL of the labeled Vol1 culture diluted into fresh AMP media. EZ55 cultures (~10 mL) were prepared in 12 dialysis bags and two were placed into each of the six dishes. The dishes were incubated in a sun box with aeration. Time zero samples were immediately sampled in triplicate by filtration from the remaining culture stocks. A *Prochlorococcus* Vol1 culture with natural abundance carbons was also sampled at this time. At 4 h, the dishes were removed from the sun box and one of the EZ55 dialysis bags from each dish was extracted. The dishes were returned until the 8 h time point was reached, when the remaining six EZ55 cultures were extracted for a total of 21 samples. For extraction of each sample, 10 mL of the culture was collected on a nylon filter and immediately transferred cell-side down to a 60 x 15 mm petri dish at $-20\text{ }^{\circ}\text{C}$ containing 1.3 mL of an extraction solvent (0.1 M formic acid in 1:2:2 water:methanol:acetonitrile) and internal standards (42.5 μM tris(hydroxymethyl)aminomethane and 17 μM benzoic acid). The samples were then allowed to extract for 20 min at $-20\text{ }^{\circ}\text{C}$. The filters were then washed by pipetting the extraction solution over the surface for approximately 30 s. The solution was then centrifuged (5 min, 13.2 rpm, 16.1 rcf, 4°C) and 300 μL of the supernatants was added to each of two autosampler vials containing 26 μL of 15% ammonium bicarbonate. One vial was placed directly in the autosampler for analysis, and the other was frozen at -80°C for analysis in the opposite mode the following day. Experiments were complete as triplicate technical replicates.

General Chromatographic, MS, and Data Analysis procedures. Completed as detailed in Chapter 2.

CHAPTER V

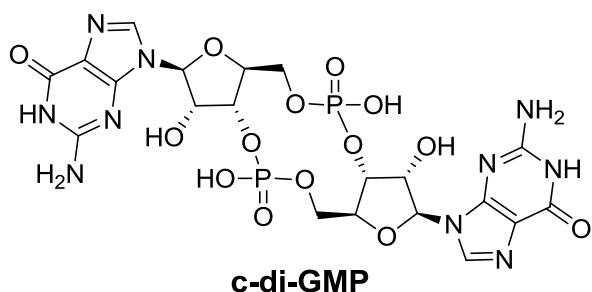
DEVELOPMENT OF A CYCLIC-DI-GMP INTERNAL STANDARD AND ITS APPLICATION TO THE UNDERSTANDING OF AZOSPERILLUM BRASILENSE SIGNALING

5.1 Introduction

Motile microorganisms monitor physical and chemical changes in their environment and can adapt by moving through physiochemical gradients in a fashion advantageous to the organism. Energy taxis describes a mechanism for coupling intracellular metabolic signals to active migration and has been described in a variety of species.¹¹⁶⁻¹¹⁹ Redox state,¹²⁰ oxygen concentrations,¹²¹ and proton motive force,^{122,123} all functions associated with the electron transport chain, are sensed by energy-taxis related proteins. The PAS domain is responsible for sensing oxygen, redox state, and light as the sensory portion of many energy taxis proteins.¹²⁴

It is known that chemotaxis related motility is inhibited by cyclic-diGMP. A diguanilate cyclase domain (GGDEF) and a phosphodiesterase domain (EAL) are common to most sequenced motile species of bacteria and often occur within the same protein. Studies have shown that these bifunctional proteins help regulate c-di-GMP concentrations and in turn motility.¹²⁵ *Azospirillum brasilense* is a nitrogen fixing α -proteobacterium isolated from the rhizosphere of cereals including rice and wheat, and is interesting for the diversity and number of its cyclic-diGMP binding domains. The genome contains 15 c-di-GMP specific phosphodiesterase (PDE) domains, 30 diguanilate cyclase (DGC) domains, and most interestingly 5 PilZ domains. One of its energy taxis transducers, tlp1, contains a PilZ domain in the cytoplasmic region. Only 33 of the greater than 12,000 annotated chemoreceptors from sequenced bacteria and archaea genomes contain a PilZ domain, and the *A. brasilense* Sp245 genome contains

five.¹²⁶ This PilZ domain binds cyclic-diGMP and is thought to function as an intracellular sensing domain of this membrane receptor. Herein we characterize the nature of c-di-GMP involvement in energy taxis.



5.2 Results and Discussion

Nine mutant strains were established based on *A. brasilense* wild type strain sp7. The first set of strains consists of a *tlp1* mutant complemented with a low copy number plasmid (pRK415) containing either full length *tlp1* (pRKTlp1), *tlp1* with PilZ deleted (pRKΔpilZ), site directed replacement of Arg562 (pRKSDM1), or both Arg562 and Arg563 (pRKSDM2) with alanine. Other mutants included *chsA*, a phosphodiesterase (PDE), and chemotaxis proteins *cheA1*, *cheB1*, and *cheBR*. *In vitro* binding studies showed that Tlp1 does bind c-di-GMP with a reasonably high affinity (K_d 6.9 ± 1.2 μ M). The mutated arginine residues belong to a portion of the c-di-GMP binding site of Tlp1. Deletion of the semi-conserved Arg562 reduced binding affinity substantially (K_d 21.8 ± 7.1 μ M), and binding disappeared when both Arg562 and Arg563 were deleted. No differences in growth phenotype was observed for any mutants.

Next, transient interactions between small groups of motile cells (<10 cells), a behavior known as clumping, were measured for each strain. In these experiments, wild type cells decrease clumping when perturbed by either a switch to a nitrogen only atmosphere from air or vice versa, but the steady state clumping recovers in 30 to 45 s. This recovery was slower in nitrogen than in air. In the Tlp1 deficient mutants, *tlp1* pRK415, *tlp1* pRKΔpilZ, and *tlp1* pRKSDM2, this recovery was delayed up to 4 min. These behaviors are reminiscent of those observed in chemotaxis mutants Δ *cheA1* and Δ *cheB1*. A PDE mutant, *chsA::Tn5*, which contained a PAS domain, was found to clump under all conditions and provided direct evidence of a link between c-di-GMP and clumping.

Measurement of c-di-GMP was attempted by MS to correlate c-di-GMP concentration and the clumping phenotype. Early MS experiments showed c-di-GMP was only observed very near the limit of detection and a [U-¹³C]-c-di-GMP internal standard was prepared enzymatically from GTP to enable better quantitation. In the course of an enzymatic synthesis of [U-¹³C]-c-di-GMP, two technical problems were encountered. First, the enzyme PleD carried large amounts of natural abundance c-di-GMP through the purification steps, and then as it released the material it contaminated the solution of [U-¹³C]-c-di-GMP. When the *E. coli* containing the PleD plasmid were

grown on media containing ($^{13}\text{C}_6$)glucose as the carbon source, the labeled form was carried through purification instead and contamination was avoided. The second technical issue arose due to the inhibition of PleD by c-di-GMP, which caused low yields. To circumvent this problem the enzyme was concentrated by ultrafiltration, which concurrently purified any synthesized c-di-GMP for analysis and prepared the enzyme for use in another synthetic reaction.

Experiments to measure c-di-GMP from cultures were carried out as follows. Cultures were bubbled with air for several minutes before being switched to nitrogen (or vice versa), and 40 mL was sampled at 10, 20, and 40 s. Such a large sample could not be filtered efficiently, as filtration was slowed and finally completely blocked by the number of cells collected. Therefore samples were added to tubes cooled with liquid nitrogen and centrifuged until just thawed. The supernatants were then removed and the pellets extracted with solvents containing internal standard. Even with the new internal standard, variability among samples was too great to discern temporal changes. However, steady state measurements of *sp7* and *chsA::Tn5*, show a significant difference in c-di-GMP concentrations between the two strains. This implies that the clumping phenotype is regulated in part by c-di-GMP concentrations.

5.3 Conclusions

The c-di-GMP binding PilZ domain is necessary for the function of the energy taxis transducer Tlp1 and for normal motility. Chemotaxis regulating proteins containing GGDEF and EAL domains are reported in the literature to control the intracellular c-di-GMP concentrations and promote biofilm formation upon increasing c-di-GMP levels, or swimming upon a decrease. Although not a perfect analogy, similar regulation appears to be at work in energy taxis, where an increase in c-di-GMP concentration is correlated with constitutive clumping in a PAS containing protein. The number of PAS domains in a genome has been correlated to number of electron transport system genes, but not to the overall size of the genome, indicating that redox metabolites, oxygen concentrations and other measures of cell state can regulate c-di-GMP levels and therefore downstream behaviors. PilZ domains of energy taxis transducers offer a link between the c-di-GMP second messenger and active migration.

5.4 Methods

Bacterial growth conditions. Cells were grown in *A. brasilense* minimal media (MMAB)¹²⁷ with 0.5% (w/v) sodium pyruvate as the carbon source replacing malate.

Clumping Assay. Cells (10 μ L drop) grown in high aeration conditions were suspended in MMAB with an added 10 mM pyruvate and placed in a gas perfusion chamber. The humidified gas flowing above the droplet was first set to compressed air, which contains 21% oxygen, for about 5 min or until equilibration. Then video recording was started, and after 1 min the gas valve was switched to nitrogen (0% oxygen). At 7 min, the valve was switched back to compressed air, and recording continues until 10 min. Using the Cell Counter plug-in from ImageJ (<http://rsbweb.nih.gov/ij/>) in conjunction with the digital video recording, the clump-to-free swimming cell ratio was determined. The number of cells forming transient cell-cell contacts (clumps) and those that were free swimming were manually counted by visual inspection (through microscopy) for a given video frame. Clips before and after the selected frame were checked for motion that indicated clumping behavior and not simply overlapping cells. At least 3 frames were counted for each measurement.

Preparation of (¹³C₂₀)c-di-GMP. Due to incorporation of endogenous *E. coli* unlabeled cyclic-di-GMP, *E. coli* BL21 cells containing the *Caulobacter crescentus* DGC protein PleD* (constitutively active) expressed from the plasmid pRP87 were grown and induced in M9 medium supplemented with (¹³C₆)glucose as the carbon source. Briefly, cells were inoculated into M9 medium from an overnight culture and grown until OD₆₀₀ reached 0.5. IPTG was added to a final concentration of 0.4 mM and cells continued to incubate at 22°C overnight. Cells were collected and resuspended in lysis buffer (25mM Tris-HCl, pH 8.0, 500 mM NaCl, 10 mM imidazole) and lysed by several passages through a French Press. Cell debris was removed by centrifugation at 17,000 rpm for 45 min. The resulting supernatant was incubated with equilibrated Ni-agarose resin by batch binding at 4 °C for 30 min and loaded onto a gravity column, washed thoroughly with wash buffer (lysis buffer with 30 mM imidazole) and eluted with 250 mM imidazole. The eluate was concentrated by ultrafiltration and dialyzed to remove imidazole prior to c-di-GMP synthesis.

Cyclic-di-GMP was synthesized using ($^{13}\text{C}_{10}$)GTP (25 μM) and aliquots of purified PleD* (30 μM) in synthesis buffer (75 mM Tris, pH 8, 250 mM NaCl, 25 mM KCl, and 10 mM MgCl_2) overnight at 25°C. Due to c-di-GMP inhibition of PleD activity, the enzyme was concentrated by ultrafiltration. The flow through was analyzed for c-di-GMP purity while the enzyme fraction was diluted in fresh synthesis buffer with an additional 25 μM ($^{13}\text{C}_{10}$)GTP for another round of synthesis. After overnight incubation, reactions were boiled to denature protein, centrifuged, and supernatant collected for c-di-GMP analysis.

Solutions were tested for the presence of any GDP, GTP or c-di-GMP containing the natural isotopes. Those that contained only ($^{13}\text{C}_{20}$)c-di-GMP were pooled and lyophilized. The powder was then resuspended and the final concentration was measured by a UV-Vis spectrometer at 254 nm with an extinction coefficient of 26,100 $\text{M}^{-1}\text{cm}^{-1}$. This stock solution (190 μM) was stored at room temperature.

Sampling *A. brasilense* for c-di-GMP. Cultures of *A. brasilense* were grown to a density of 0.7 OD_{600} and then aerated cultures with air for several minutes. Approximately 45 mL of the culture (Air 0) was drawn into a syringe and delivered into a 50 mL conical tube pre-cooled in liquid nitrogen. This tube was capped and kept on liquid nitrogen until sampling was finished. The gas was then switched to nitrogen only and sampled after 10, 20 and 40 s as described above. The culture was allowed to acclimate to nitrogen for 6 minutes. Then a steady state nitrogen aliquot was sampled before switching the gas back to air. Again samples were taken at 10, 20 and 40 s after perturbation with air. Samples were allowed to stand on the bench top for a minute or so before centrifuging for 30-45 min, until cultures are completely thawed and pelleted taking care that as little time thawed is allowed as possible to prevent enzymatic degradation of c-di-GMP. Supernatants were then decanted and the pellets were resuspended in 750 μL extraction solvent containing benzoic acid (17 μM) and ($^{13}\text{C}_{20}$)c-di-GMP (0.76 μM). The samples were incubated at 4 °C for 30 min to complete extraction. The extracts were then transferred to microcentrifuge tubes, and centrifuged at 4 °C on max speed for 15 min. A portion of the supernatants (300 μL) was then transferred to another microcentrifuge tube containing 26 μL 15% w/v ammonium bicarbonate, at which time a precipitate was observed. These were also centrifuged at 4 °C on max speed for 15 min then transferred to an autosampler vial and stored at -80C until MS analysis.

Protein concentration assay. Protein concentrations were assay by the Bradford method from a 1 ml aliquot of the culture immediately following sample collection.

Mass Spectrometry Analysis. Chromatography was completed as described in chapter two for negative mode, and MS analysis was completed using the general methods described there. Two SRMs were previously reported for c-di-GMP (689-344 and 689-150).⁵⁹ The equivalent reaction for (¹³C₂₀)c-di-GMP was obvious for the first SRM because it described fragmentation to a single GMP (709-354) and as this was also the most SRM this pair was chosen for quantitation.

Data Handling . Peaks were integrated manually in Xcalibur QualBrowser and the concentration of c-di-GMP was calculated using the following equation:

$$\frac{cdiGMP\ signal}{(^{13}C_{20})cdiGMP\ signal} * known\ (^{13}C_{20})cdiGMP * \frac{1}{[protein]} * \frac{1}{volume\ sampled} *$$

$$volume\ extraction\ solvent = \frac{[cdiGMP]}{Protein}$$

CHAPTER VI

INTRODUCTION TO QUORUM SENSING

6.1 Bacterial Communication

A few words are necessary on the mass-action of bacteria. It is a common observation, one made by the writer at least a hundred times, that in culture-media not exactly adapted to the needs of the organism, a scanty inoculation may not give any growth—not even after a long time—whereas a copious one will lead to a growth which gradually clouds the fluid or covers the solid. The penetration of bacterial strands from cell to cell in the root nodules of Leguminosae is another example. The only explanation I can think of is that a multitude of bacteria are stronger than a few, and thus by union are able to overcome obstacles too great for the few. Dr. Erwin F. Smith, 1905, Bacteria in Relation to Plant Disease, Vol. 1, Carnegie Institution Report.

As illustrated by the words of Erwin Smith, scientists have long appreciated that bacteria demonstrate group beneficial behaviors. Many phenotypes display this group psychology including pathogenesis and biofilm formation, and therefore gaining a more complete understanding of this phenomenon is appealing to a number of industries. Among these are pharmaceuticals and health care, as disruption of these coordinated behaviors may lead to better outcomes for patients with cystic fibrosis,¹²⁸ medical implant,¹²⁸ and infectious diseases.¹²⁹

It follows that these behaviors must be regulated by a means of communication. Broadly, bacteria can communicate through two mechanisms either by direct-contact using adhesions and surface receptors or by diffusible chemical signals.¹³⁰ However it was not until the 1970s that the diffusible mechanisms of communication were discovered and many open questions on the subject remain today.

6.2 Quorum Sensing

The use of small diffusible molecules to regulate cell density dependent gene expression is referred to as quorum sensing.¹³¹ These signaling molecules are made by bacteria to induce particular phenotypes independent of growth, and therefore are designated “autoinducers.”¹³¹ There are three major classes of quorum sensing signals: peptide autoinducers (AIPs),¹³² acyl-homoserine lactones (AHLs),¹³³ and derivatives of

4,5-dihydroxy-2,3-pentanedione (DPD) collectively called autoinducer-2 (AI-2),¹³⁴ although other classes of molecules are sometimes used in the same fashion, such as PQS of *P. aeruginosa*¹³⁵ and CAI-1 of *V. cholera*¹³⁶ (Figure 6.1). A bacterium will produce a proportional amount of autoinducer, which diffuses and externally builds in concentration as the bacterium divides and more bacteria accumulate and produce their own autoinducer. Once a threshold concentration of autoinducer is reached, the bacteria turn on certain phenotypes in a coordinated fashion.¹³⁷ The quorum sensing regulated behaviors are typically those which would be unproductive if carried out by an individual. For example, once a pathogenic bacterium enters a host system, it faces two formidable obstacles: first, it must compete with a large number of bacteria that have already colonized the host, and second, it must avoid the host immune system. Therefore, attracting attention by expressing virulence factors is likely to end in destruction of the bacterium. Alternatively, if the bacterium can find a niche to start growing and dividing and wait until a small army of bacteria is available to launch a coordinated attack, the pathogens are much more likely to succeed and survive.

6.3 Peptide Autoinducer Signaling

The first clear evidence of cell-cell communication among a community of bacteria came in the mid-1960s.¹³⁸ A “hormone-like” activator was discovered that controlled the ability of bacteria to incorporate exogenous DNA, called competence, in several species of *Streptococci*. Alexander Tomasz is often credited with the discovery, as he recognized “the induction process seems to be a novel phenomenon among bacteria” and extrapolated the idea saying, “One wonders whether this kind of control may not be operative in some other microbial phenomena also.” However, he credits several contemporaries¹³⁹⁻¹⁴¹ also studying group behaviors of *Streptococci* and *Bacilli*. One of the important achievements of the paper was the demonstration that activator production was independent of nutrient availability and cell cycle, which addressed the conventional explanation for synchronized cultures. Then Tomasz was able to show that competent cells could synchronize non-competent cells. The final key success was the demonstration that the effect would take place without contact between cells, therefore indicating a diffusible signal was responsible. While the original letter contained evidence

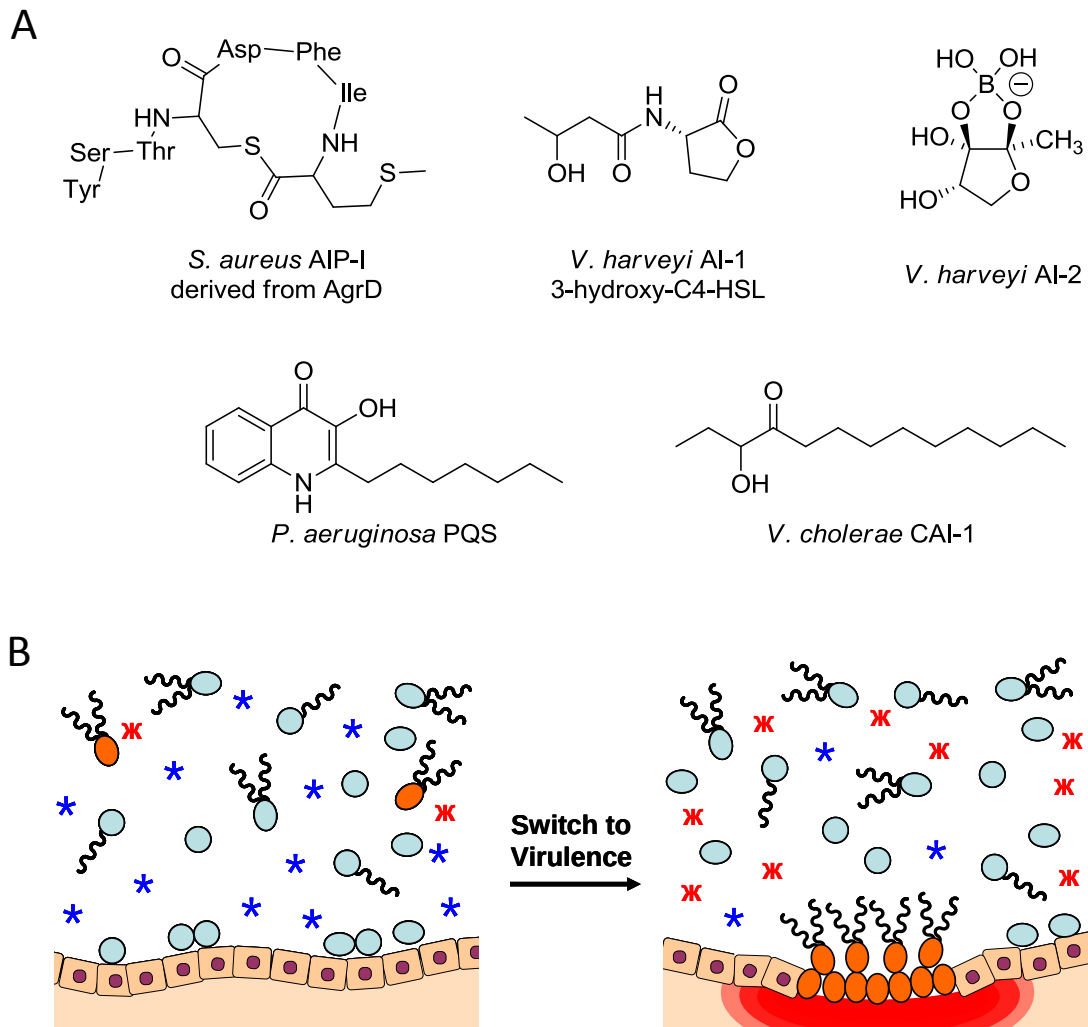


Figure 6.1. A) Representative members of the three major classes of quorum sensing signals and two quorum sensing signals that do not fit these categories. B) An illustration showing the biological relevance of quorum sensing.

that the activator for *S. pneumoniae* was a protein and proposed a chromatographic method for its purification, it was not until 1995 that the structure was identified.¹⁴² Surprisingly, the structural identification was done by Edman degradation, a method developed in 1950 to sequence peptides by reacting the N-terminus with phenylisothiocyanate.¹⁴³ The activator was identified as a heptadecapeptide peptide now called ComC.

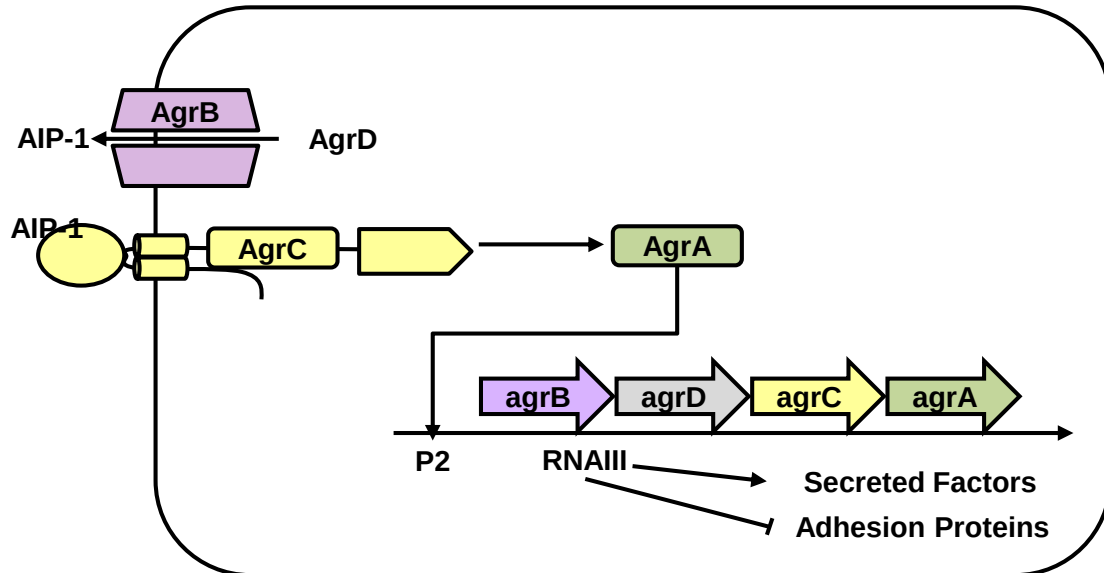
Another famous example of peptide quorum sensing is the *agr* system of *Staphylococcus aureus*.¹⁴⁴ The *agr* system controls the expression of K-, L- and N-toxins, serine protease, DNAase, fibrinolysin, enterotoxin B and toxic shock syndrome toxin-1 virulence factors. The locus is comprised of five genes: the components of a two-component regulatory system AgrA and AgrC, the propeptide AgrD, an AgrD transport and processing protein AgrB, and an RNA regulatory molecule, RNAIII, that also encodes N-toxin. The propeptide AgrD is processed to form an octapeptide containing a thiolactone, which interacts with the AgrC receptor. AgrC is autophosphorylated, and then phosphorylates AgrA which activates the transcription of the *agr* operon, thus earning the name autoinducer (Figure 6.2). Significantly, a similar *agr* system has been implicated in biofilm formation occurring in *S. epidermidis* infections. Interestingly, the peptide was found to be specific to group of *Staphylococci*, and several other species of *Staphylococci* produce a similar signal each of which can inhibit the effects of the others.¹⁴⁵

The signals ComC and AgrD are just two examples of an ever growing vocabulary of peptide autoinducers, which can range from a few amino acid residues to more than 30 kDa. AIPs are typically specific to a particular species of bacteria or a small subset of species within a genus, and a large number of them are antibiotic in nature. The AIP systems are primarily found in gram-positive bacteria, although several gram negative bacteria have been known to make them. A further discussion of AIPs can be found in reviews by Shapkov and Abraham.

6.4 Acylhomoserine Lactone Signaling

In 1970 Nealson and coworkers described another system of cell-cell communication, this time in an obscure gram-negative species of marine bacteria, *V. fischeri*.^{146,147} This bioluminescent species colonizes the light organ of

A



B

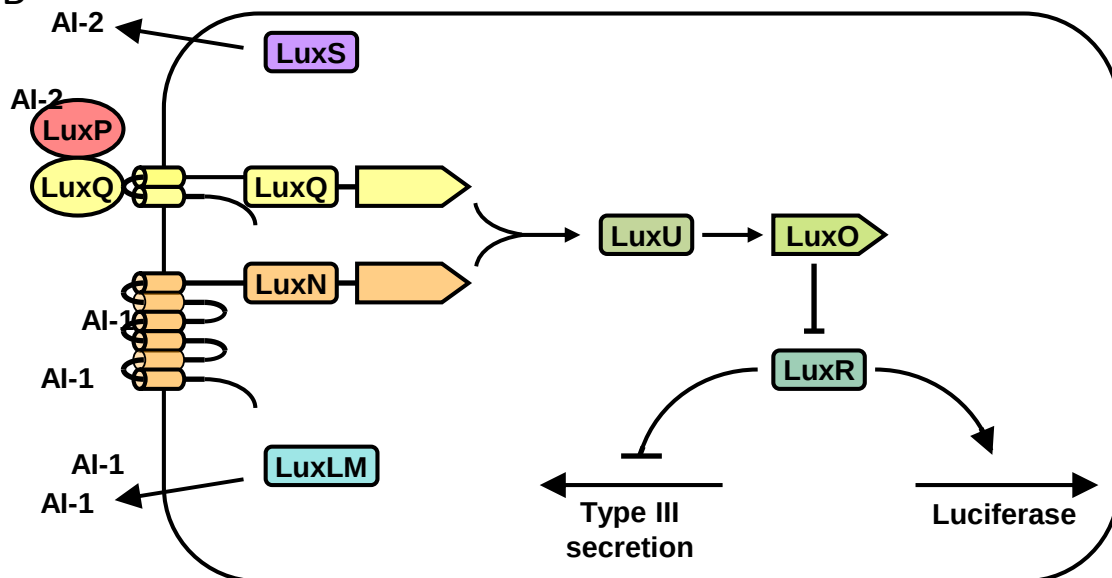


Figure 6.2. Quorum sensing pathways in A) *S. aureus* and B) *V. harveyi* representing one well characterized system for each of the three major classes of quorum sensing molecules.

the Hawaiian bobtail squid (*Euprymna scolopes*) during the day, and luminesces at night. This behavior provides protection to the squid by imitating moonlight and preventing its silhouette from being observed by predators below.¹⁴⁸ Nealson had observed that the expression of bioluminescence did not occur in concert with culture growth, but instead was only expressed when mid–logarithmic phase was reached. An investigation of likely regulatory mechanisms led the team to conclude that luminescence was regulated by a diffusible signal at the transcription level. The signal was later identified as a homoserine lactone with an amide linked 3-oxohexanoyl acyl group.¹⁴⁹ The acyl-homoserine lactone (AHL) motif was found to be a common theme among quorum sensing proteobacteria, although the number and variety of side chains employed is characteristic to the bacterial species.¹⁵⁰

These signals perform a variety of functions ranging from symbiosis, to pathogenesis, to metabolism. For example, most bacteria will initially excrete acetate as a byproduct of metabolism, acidifying the growth medium in the process. In later growth phases, the bacteria begin consuming the acetate for food, and this metabolic change is known as the acetate switch. The C8-AHL synthase in *V. fischeri*, AinS, is known to regulate this switch.¹⁵¹ The majority of this dissertation will focus on the final major signal class, AI-2.

6.5 A Dual Quorum Sensing System in *V. harveyi* and the Discovery of Autoinducer-2

The signaling molecule autoinducer-2 (AI-2) was originally identified in *V. harveyi* by Bassler and coworkers while studying the mechanisms regulating homoserine lactone (HSL) production and bioluminescence.^{152,153} Transposon insertion screening for Lux⁻ (dark) phenotypes failed to identify the *V. harveyi* HSL synthase. Additionally, transcription of the light production operon in *E. coli* was not cell-density dependent as it was typically observed in *V. harveyi*, nor was it responsive to the addition of autoinducer. These results indicated that there may be another means of activating light production, thereby preventing a dark phenotype for the HSL synthase mutant. The components responsible for cell-density regulation and HSL sensing were also missing as evidenced by the recombinant *E. coli* experiments.¹⁵² These data led to speculation that there was

another autoinducer system and with these clues, further investigation of dim transposon mutants led to the identification of LuxP and LuxQ, the two component signaling system for AI-2.¹⁵³ In the process of identifying the autoinducer-2 system, a mutant with a HSL sensor/AI-2 sensor⁺ (luxN⁻/luxQ⁺) phenotype was found, which allowed for the screening of other mutants incapable of producing AI-2.¹⁵³ The luxQ⁻ (BB886) and luxN⁻ mutants (BB170) were also used to test other species for autoinducer production. The first study of this type identified five vibrio species and two unrelated species that produced AI-2. This stood in sharp contrast to the one non-harveyi species found to produce AI-1 in 26 strains studied.¹⁵⁴ With this evidence in hand, AI-2 was hypothesized to be an interspecies quorum sensing molecule.

6.6 Biological Diversity of AI-2 and Signaling Uses

After identification and sequencing of the AI-2 synthase (LuxS) in 1999,¹⁵⁵ sequenced genome databases could be searched for *luxS* orthologues to determine the breadth of biological diversity capable of making AI-2 and trace its evolution.^{156,157} These studies found evidence of an AI-2 synthase in 55 sequenced bacteria across six phyla (deinococcus-thermus, proteobacteria, firmicutes, actinobacteria, spirochaetes and bacteroidetes), which amounted to almost half of the sequenced bacteria at the time. These findings lent support to the idea that this signaling system could facilitate inter-species communication. Many bacteria were then screened for activity in the *V. harveyi* bioluminescence assay¹⁵⁸⁻¹⁶⁴ and for phenotypic changes in *luxS* mutants.^{159,160,162-164} These studies were largely hindered by the lack of synthetic AI-2 necessary to show the phenotype could be recovered by the signal. Critics of these studies argued that the mutation could cause a metabolic disruption,¹⁶⁵ and the resulting phenotype was not necessarily due to interruption of cell-cell communication. Further they argued, the conditioned media being used to add AI-2 activity to a culture was full of many other molecules including nutrients, and until a molecularly defined solution was used, no conclusions could be made from these studies.¹⁶⁵ Thus the field was largely held back by the need for a synthetic source of AI-2.

AI-2 activity was known to arise from LuxS cleavage of S-ribosylhomocysteine (SRH) as early as 2001.¹⁶⁶ However the biologically active structure of AI-2 was not identified until 2002, when the *V. harveyi* periplasmic receptor LuxP was crystallized with the

molecule bound in the form of a borodiester.¹⁶⁷ When LuxS cleaves SRH, the products homocysteine and S-4,5-dihydroxy-2,3-pentanedione (DPD) are formed. DPD exists in equilibrium in aqueous solution in many forms since it is capable of cyclization and hydration.¹⁶⁸ The molecule is also capable of forming boroeester bonds spontaneously in the presence of boric acid, including the one characterized by x-ray crystallography¹⁶⁸ (Figure 6.3). The multiple forms makes the molecule difficult to work with and detect, but it does explain why its identity eluded scientists for so long. Three syntheses of DPD have been reported;^{169,170,171} and of these, the Semmelhack method¹⁶⁹ is utilized in our lab because it includes a means for introducing a stable isotope, unlike the De Keersmaecker synthesis,¹⁷¹ and is superior in efficiency to the Meijler route.¹⁷⁰

With a synthesis of DPD in place, microbiologists began to revisit AI-2 signaling in individual species, as well as multispecies systems. A study of two early colonizers of tooth enamel, *Streptococcus oralis* and *Actinomyces naeslundii*, was able to show that this dual-species biofilm system would not form if *luxS* was removed from *S. oralis*.¹⁷² When the cultures were complemented with synthetic DPD the phenotype was rescued at 0.8 nM DPD, but not at lower or higher concentrations. This remains one of the most compelling arguments in the literature that AI-2 signaling occurs outside of *V. harveyi* and that it mediates inter-species signaling in a biologically relevant manner.

6.7 *S. typhimurium*, *E. coli* and the Metabolism Hypothesis

Salmonella typhimurium and *E. coli* respond to autoinducer-2 very differently than *V. harveyi*. The DPD recognition protein is not a receptor, but rather an ABC transport complex, made up of the proteins LsrA, LsrB, LsrC, and LsrD.¹⁷³ The extracellular portion of this transporter, LsrB, has homology with the ribose binding protein in *E. coli* and the galactose binding protein in *S. typhimurium*.¹⁷³ LsrB also binds a nonborated AI-2 molecule cyclized with the opposite stereochemistry of the AI-2 bound by *V. harveyi*.¹⁷⁴ Transcription of this transport complex is de-repressed by the presence of phosphorylated AI-2. The transporter imports extracellular AI-2, where it is then phosphorylated by LsrK and degraded by LsrF, LsrG, and LsrE in the case of *S. typhimurium* (Figure 6.4).¹⁷⁵ No other genes are known to be regulated by AI-2 in *E. coli*. Together, *V. harveyi* and *E. coli* represent the best characterized species of the two major classes of AI-2 response pathways.

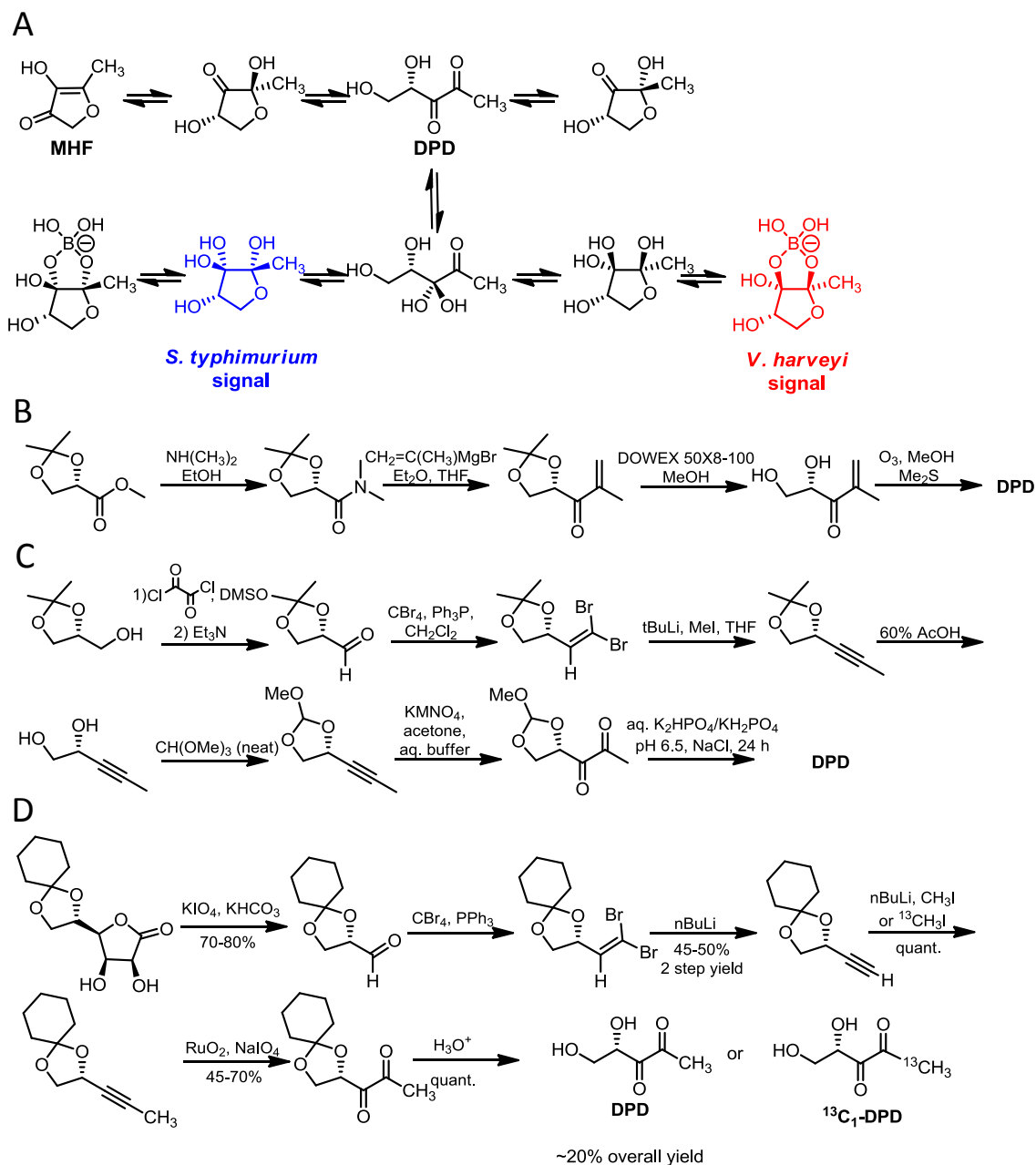


Figure 6.3. DPD chemical properties and synthesis. A) DPD exists in equilibrium with its hydrated and cyclized forms, collectively called AI-2. The DPD syntheses of B) De Keersmaecker and coworkers C) Meijler and coworkers and D) Semmelhack and coworkers.

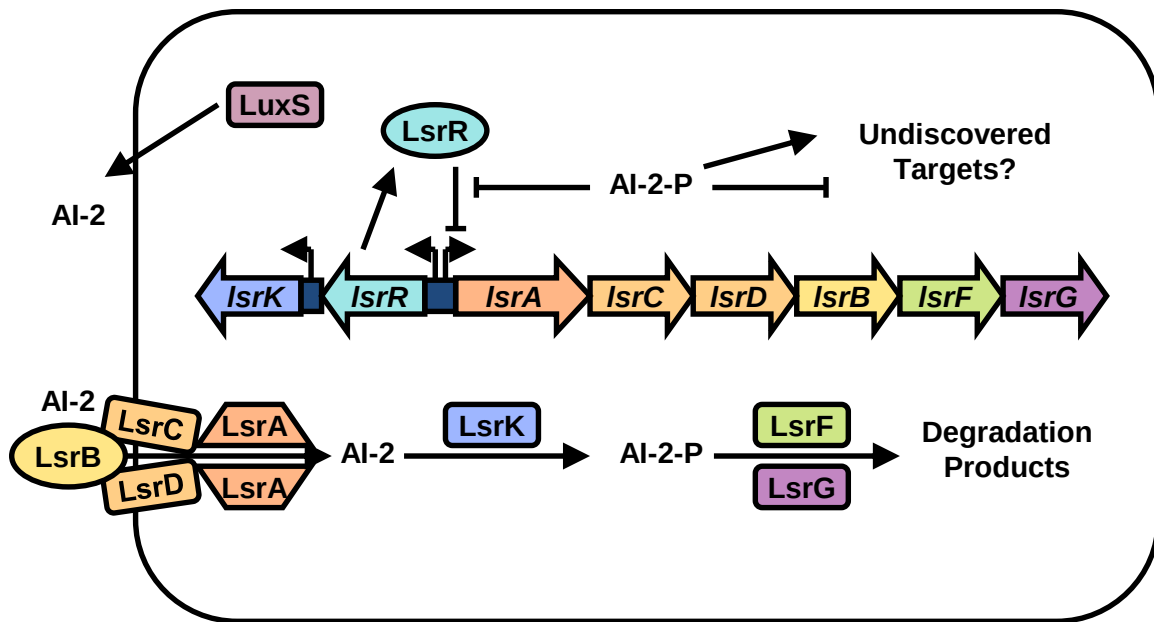


Figure 6.4. In *E. coli* and *S. typhimurium*, AI-2 de-represses transcription of proteins for its importation and degradation.

The lack of gene transcription targets and this novel mechanism for the rapid importation of AI-2 led to the hypothesis that these species do not use AI-2 signaling themselves, but rather import AI-2 as a defense mechanism.¹⁷⁵ Others believe that AI-2 is produced solely for metabolic reasons, and the import mechanism is a means to recover lost carbon building blocks.¹⁶⁵ After all, an ABC transport complex binding a small sugar, importing it, and phosphorylating it before further catabolism is reminiscent of other nutrient scavenging mechanisms.

More importantly, LuxS was actually characterized in the 1968 by Miller and Duerre as S-ribosylhomocysteinease which is part of a bacteria-specific modified activated methyl cycle (AMC).¹⁷⁶ The activated methyl cycle begins with the conversion of methionine to S-adenosylmethionine (SAM), a major cellular source of activated methyl groups (Figure 6.5). Upon transmethylation, SAM is converted to S-adenosylhomocysteine (SAH), which can be converted to homocysteine through one of two routes. In most cases, SAH is hydrolyzed in one step to adenosine and homocysteine. In many bacteria however SAH is recycled in two steps. First the adenine is cleaved to produce S-ribosylhomocysteine (SRH), and then LuxS cleaves SRH into homocysteine and DPD. At first, the production of DPD from this reaction is not obvious.

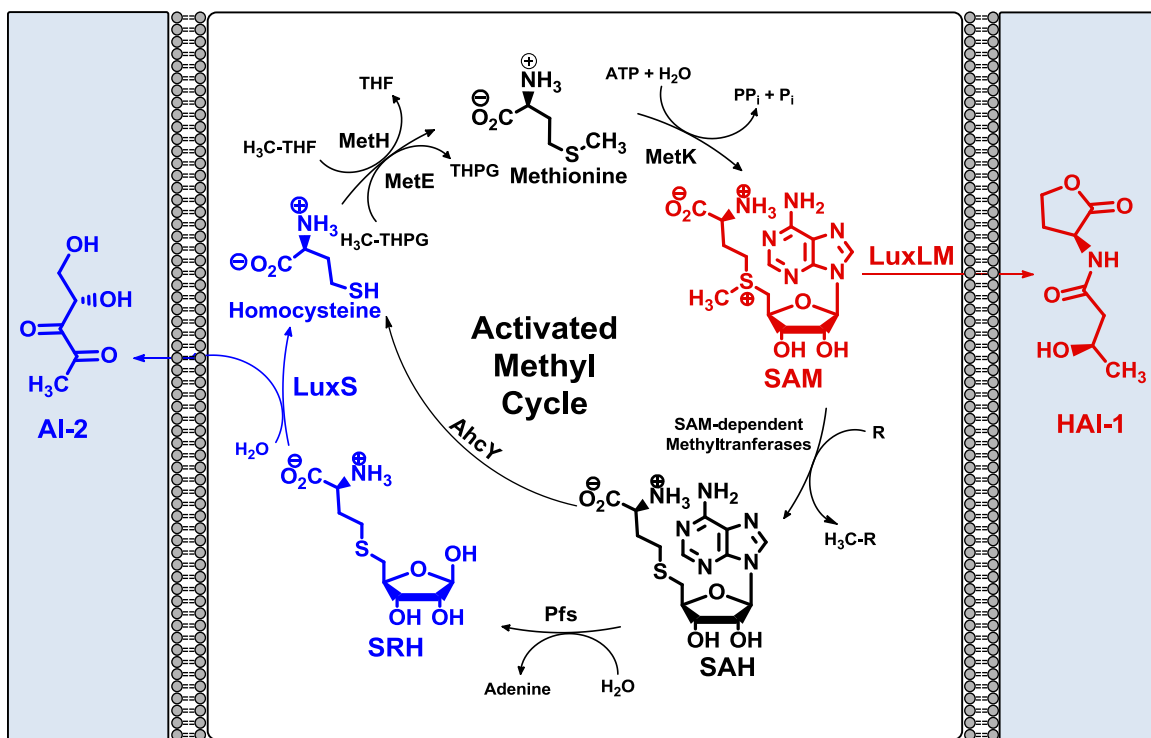


Figure 6.5. The activated methyl cycle contains the AI-2 synthase LuxS.

The enzyme mechanism utilizes an iron ion to activate the carbonyl moieties and cysteine and glutamate residues to facilitate proton exchanges. Two Lobry-de Bruyn-van Ekenstein-like rearrangements are facilitated by the enzyme to shift the carbonyl to carbon three. Elimination of homocysteine, followed by tautomerization complete the process.¹⁷⁷

SAM dependent methyl transferases are negatively regulated by SAH.¹⁷⁸ Therefore, the toxicity of a broken AMC is thought to conserve *luxS* in the genome. Standing in contrast to this hypothesis is the fact that *luxS* mutants are viable and in many cases demonstrate no phenotypic growth changes.^{165,175} It has also been proposed that AI-2 is allowed to diffuse out of the cell because two elimination products of DPD, MHF and DMHF, cause DNA-damage.¹⁷⁹

6.8 Open Questions

At the time we started working in the field of quorum sensing, there were several open questions. The dual quorum sensing system in *V. harveyi* is the source of many

questions. Why have more than one quorum sensing signal? Do they carry the same information, and if not what is the function of the other signal? Are the bacteria capable of integrating these two pieces of information in a meaningful way? Similar questions can be raised for bacteria that produce more than one AHL. The quorum signaling function of autoinducer-2 was largely debated too. If the molecule was only used as a signal in some species, why continue to produce it, especially if the product is thought to be toxic? If it is for metabolic reasons, how do bacteria compensate for this disruption when *luxS* is deleted in order to grow on pace with wild type bacteria? Each of these questions is an open area of research in our laboratory. To answer these questions at a molecular level, an AI-2 detection method was required, and this is the first challenge we approached.

CHAPTER VII

DEVELOPMENT OF AN AI-2 DETECTION METHOD AND ITS VALIDATION IN COMPLEX SAMPLES

The data presented below has been adapted from the following published journal article:

Shawn R. Campagna, Jessica R. Gooding, and Amanda L. May. Direct Quantitation of the Quorum Sensing Signal, Autoinducer-2, in Clinically Relevant Samples by Liquid Chromatography – Tandem Mass Spectrometry. *Analytical Chemistry* **2009**, 81 (15), 6374-6381. The project design was led by SRC. ALM designed the final syntheses, prepared the tags, and ran the kinetic studies. JRG formulated the initial synthetic strategy, performed the MS testing of the tags, and the bacteria and saliva cultures. DOI: [10.1021/ac900824j](https://doi.org/10.1021/ac900824j). Copyright © 2009 American Chemical Society

7.1 Background

While there has been great interest in characterizing the biological role of AI-2, a method for its detection in complex media has not yet been described. AI-2 exists in equilibrium with several related molecules¹⁸⁰ and this hinders detection. Additionally, the molecule cannot be measured directly due to its oligomerization upon concentration,^{168,170} poor ionization, and lack of a chromophore.

There are currently three classes of methods used to detect AI-2. The assay used in the majority of the quorum sensing literature is the *V. harveyi* bioluminescence assay.¹⁵⁴ The assay measures light induction in a reporter strain with mutations in both the HAI-1 receptor LuxN and in the DPD synthase LuxS in the presence of candidate AI-2 containing cell-free supernatants. Since the behavior of a complex biological system is central to this assay, predictably the concentration of molecules in the supernatants, e.g. glucose and boric acid, affects the assay. While this assay is sensitive and has a low nM limit of detection, its small linear range (0.08-0.8 μ M) limits quantitation.¹⁸¹ There have also been reports of low reproducibility.¹⁸²

The second type of AI-2 detection method is a sensor derived from the AI-2 receptor protein LuxP.^{183,184} The sensors are designed to take advantage of a conformational change upon binding AI-2 that moves two chromophores of a FRET pair, which subsequently changes the emission spectra. The assay has advantages over the

bioluminescence assay because the sensor is a protein instead of a complex organism, however it is still affected by the concentrations of other molecules. In particular, borate concentrations are essential to the assay since the sensor protein is derived from the *V. harveyi* receptor LuxP which binds the borate ester form of AI-2. Additional drawbacks to this method include low sensitivity limited by the signal-receptor binding affinity (270 nM), the difficulty and expense of obtaining the sensor protein, and a limited dynamic range (0.1 - 30 μ M).¹⁸³

The third category utilizes either a GC/MS or LC/MS method to detect a DPD derivative. The method reported below falls in this category and was developed concurrently to that reported by Thiel and coworkers.¹⁸⁵ Their method requires several reaction steps (Figure 7.1), followed by solid-phase extraction and 50-fold concentration, which demands extra time and handling. The method uses an internal standard that is susceptible to matrix effects and has only been validated in defined minimal media.

DPD concentrations have been measured using the FRET approach under normal laboratory conditions in the μ M range for *V. harveyi* (4.2 μ M),¹⁸³ *E. coli* (120 μ M),¹⁸⁴ *E. faecalis* (35 μ M),¹⁸⁴ *S. epidermidis* (12 μ M),¹⁸⁴ and *B. subtilis* (17 μ M).¹⁸⁴ The GC/MS method similarly measured the concentration in *V. harveyi* at 1.95 μ M DPD.¹⁸⁵ However studies of mutualistic biofilm formation in *Streptococcus oralis* and *Actinomyces naeslundii* found relevant concentrations of AI-2 to be 135-197 nM.¹⁸⁶ Unfortunately, quantitation of AI-2 at these biologically relevant concentrations is not possible using the LuxP FRET assay due to low sensitivity. In actuality, another method of quantitation is needed because each of these methods are insufficient as they are not easily reproduced, not sufficiently quantitative, capture only one form of autoinducer-2, difficult to perform, sensitive to changes in the biological environment or a combination thereof.

7.2 Experimental Design

We chose to develop an SRM based LC-MS/MS method for several reasons. MS detection is comparatively sensitive and has a large linear dynamic range. In addition, SRM technology affords three levels of specificity: retention time, parent ion m/z , and fragment ion m/z . These levels of specificity allow differentiation between as similar molecules as citrate and isocitrate and ortho- and para-aminobenzoic acid.^{20,30}

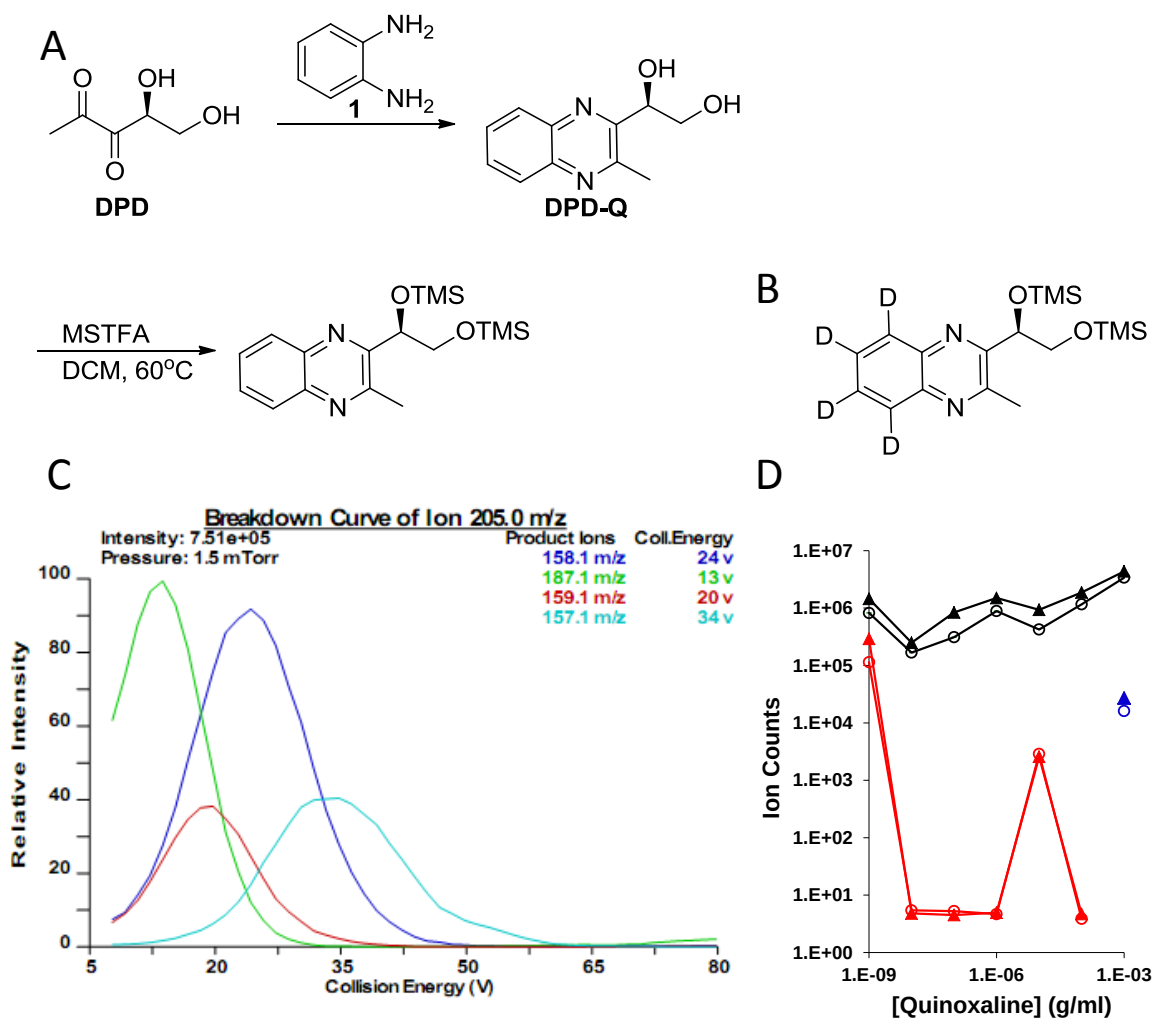


Figure 7.1. Quinoxaline formation from DPD and o-diaminobenzene, **1** (A) Derivatization of DPD via methods of Thiel and coworkers. (B) Internal standard used by Thiel and coworkers (C) Results of initial experiments with o-diaminobenzene showing poor fragment sensitivity for data collected using a 1 mg/mL solution of DPD-Q (D) Early experiments with 4,5-dimethoxy-2,3-diaminobenzene also failed due to poor sensitivity and dynamic range. Data collected for two SRMs 265-247 (▲) and 265-218 (○) injected in either water (black), cell-free supernatants (red) or through an aminopropyl column (blue).

Our candidate derivatizing agents were designed to meet the following conditions: 1) sensitive ionization in the MS 2) facile fragmentation without fragmentation in the ion source 3) C2 symmetry to assure a single product is formed, and 4) selective and expedient reaction with DPD. We hypothesized that the α -diketone of DPD was sufficiently reactive and unique to be a good handle for derivatization. Its reaction with *o*-diaminobenzene was known,¹⁷⁰ and we hypothesized that formation of the aromatic quinoxaline would be robust enough to drive the reaction to completion quickly and be stable enough for storage.

Initially commercially available and easily accessible derivatizing agents were tested. Preliminary experiments with *o*-diaminobenzene found that the corresponding quinoxaline was not detectable at concentrations less than 100 $\mu\text{g/mL}$ in positive mode and did not ionize in negative mode. Fragmentation at 1 mg/mL concentrations only produced fragment signals at 10^5 ion counts, where we would normally expect these concentrations to overwhelm the detector. A molecule with such a high limit of detection (LOD) would not be useful for detection at the very low concentrations we desired. Early results from a 4,5-dimethoxy-1,2-diaminobenzene derivatizing agent had a small dynamic range and was not detectable in supernatants, so it was also set aside (Figure 7.1 panel D).

We therefore set about designing diaminobenzene derivatives that would meet the ionization and fragmentation criteria. We hypothesized that an ether linked carbon chain with a terminal acid would enable facile fragmentation via either decarboxylation or reverse Michael addition. The first series of candidate molecules is shown in Figure 7.2 panel A. In addition, 2,3-diaminobenzoic acid was easily accessible and tested although problems arising from the formation of regioisomers were anticipated.

7.3 Synthesis of Candidate Derivatizing Agents

The synthesis for molecules **2** and **3** was straight forward (Figure 7.2 panel B). Reduction of commercially available 2-amino-3-nitrobenzoic acid by hydrogen and palladium on carbon was previously reported, and the procedure was only modified to replace methanol as a solvent with ethanol to avoid risk of explosion. Esterification of **2** using thionyl chloride and ethanol required higher temperatures than refluxing allowed, however conducting the reaction at 100 °C in a pressure vessel produced **3** in satisfactory yield (44%).

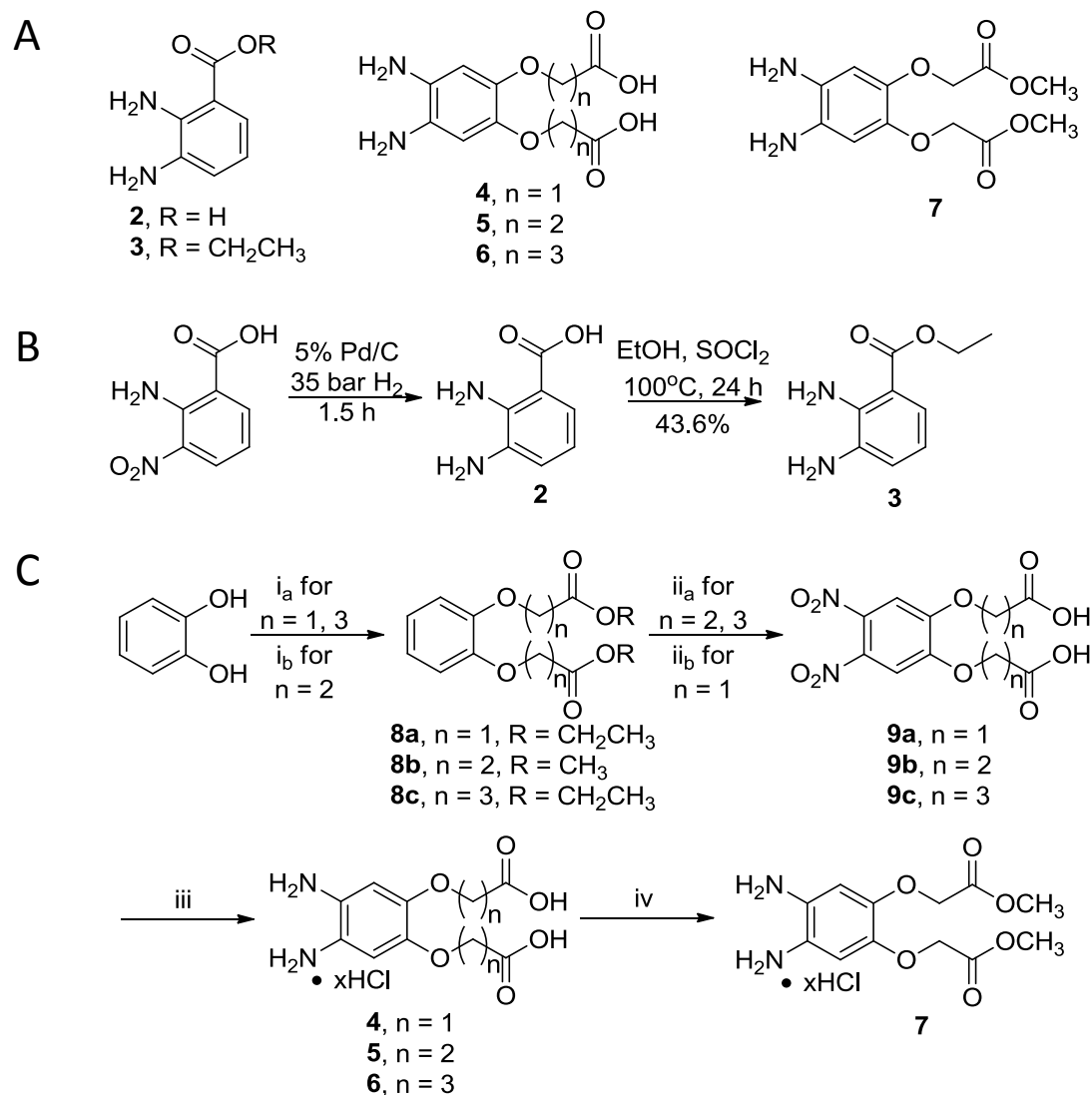


Figure 7.2. Synthesis of DPD tagging reagents (A) Molecular structures of the DPD tagging reagents used in this study. (B) Synthesis of molecules **2** and **3**. (C) Synthesis of molecules **4-7**. Reagents and conditions (i_a) catechol, acetone, Na₂CO₃, ethyl bromoacetate (n = 1) or ethyl-4-bromobutyrate (n = 3), reflux, 48 h, 48.5% for **8a**, 79% for **8c**; (i_b) catechol, methyl acrylate, NaOCH₃, 100°C, 24 h, 4.72% for **8c**; (ii_a) AcOH, HNO₃, 3% H₂O, 50°C, 24 h, 59.5% for **9b**, 96% for **9c**; (ii_b) H₂SO₄, HNO₃, 50°C, 24h, 63.6%; (iii) HCl, SnCl₂, then H₂S, quantitative for **4**, 50.4% for **5**, 40.8% for **6**; (iv) SOCl₂, MeOH, 93%.

Table 8.1 Most Sensitive SRMs for Each Quinoxaline

The route to molecules **4-7** followed the same general scheme. Catechol was substituted by addition to ethylbromoacetate in excess potassium carbonate ($n=1$), methyl acrylate in catalytic sodium methoxide ($n=2$), or ethyl-4-bromobutyrate in excess potassium carbonate ($n=3$). The electrophilic aromatic substitution of the benzene ring was then accomplished by adding nitric acid to a solution of **8a-c** in either sulfuric ($n=1$) or acetic acid ($n=2,3$), which yielded a crystalline solid **9a-c**. The nitro groups were then reduced to amines by stannous (II) chloride in HCl followed by hydrogen sulfide to precipitate the tin. The reaction gave molecules **4-6** as a purple solid in 41% ($n=3$) to quantitative ($n=1$) yield. Finally, addition of thionyl chloride to a chilled solution of **4** in methanol followed by refluxing for 1 h produced **7** in 93% yield. The entire synthesis of **7** can be accomplished in 4 days in 29 % yield with no chromatography.

7.4 Choosing a Derivatizing Agent

With these seven molecules in hand, the each molecule was mixed at a 3:1 molar ratio with DPD (7 mM tag and 2.35 mM DPD in 50 μ L H₂O) and allowed to react for 2 h to form the corresponding quinoxaline (2.35 mM theoretical yield). Each solution was diluted to 10 μ M quinoxaline, assuming the reaction ran to completion, and the four most sensitive SRMs for each quinoxaline were determined by observing the m/z when the solution was directly injected in the MS (Table 7.1). The lack of expected fragmentation ions was immediately obvious. No decarboxylation fragments were observed for the quinoxalines formed from **2** and **4-6**, nor were reverse Micheal addition fragments found for quinoxalines formed from **4** and **7**. We had also predicted that these dicarboxylic acids would ionize best in negative mode, however the positive mode SRMs were the most sensitive. In spite of these results, all quinoxalines were carried into the next set of experiments since they were each detected and each fragmented easily. The relative ionization efficiencies of the quinoxalines were tested by injection onto an aminopropyl column using a gradient modified from our standard metabolomics method.

The quinoxalines generated by reaction with molecules **4-6** showed at most 10% of the signal intensity of the other three (Figure 7.3). The greatest intensity was observed for the quinoxalines formed from **3** (**DPD-EBAQ**); however, the peak observed was asymmetric presumably due to partial separation of the two regioisomers. Similarly, a

Table 8.1 Most Sensitive SRMs for Each Quinoxaline

Quinoxaline	Parent Ion (<i>m/z</i>)	Product Ion (<i>m/z</i>)	Collision Energy (V)
DPD-BAQ	249	144	35
		173	27
		201	24
		231	17
DPD-EBAQ	277	144	36
		173	29
		201	25
		231	17
DPD-M1CQ	381	201	36
		202	43
		231	29
		363	17
DPD-1CQ	353	175	31
		202	42
		231	30
		335	18
DPD-2CQ	381	272	23
		291	31
		291	24
		363	14
DPD-3CQ	409	190	49
		219	28
		237	25
		305	20

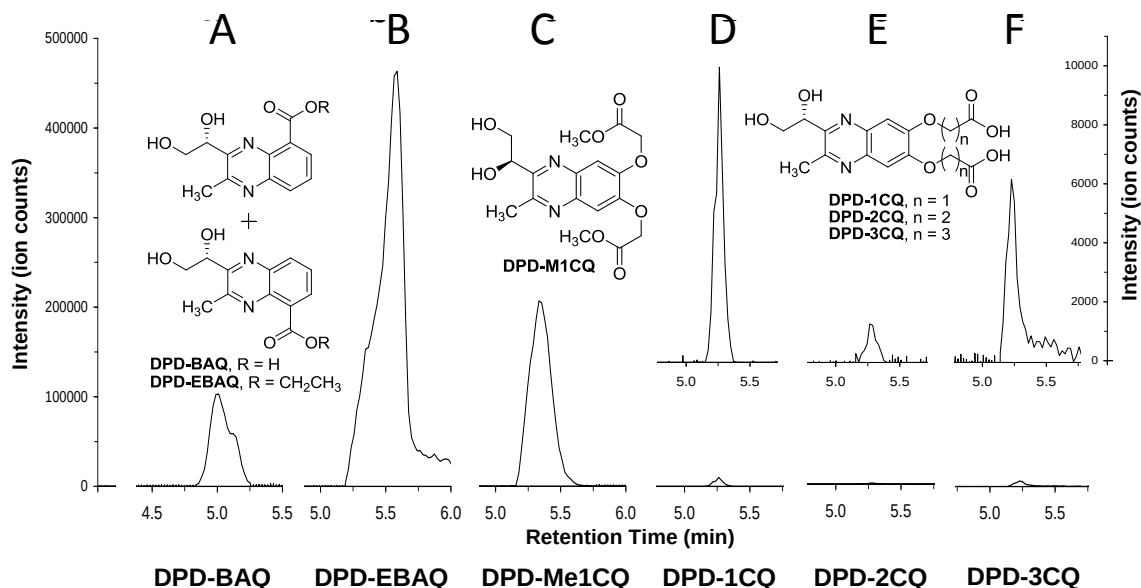


Figure 7.3. Mass Chromatograms for each **DPD** derived quinoxaline. A 10 μ L injection of a 10 mM solution for each was detected by an appropriate SRM after gradient elution from an aminopropyl LC column. (A) **DPD-BAQ** as detected via SRM 249-231. (B) **DPD-EBAQ** as detected via SRM 277-231. (C) **DPD-M1CQ** as detected via SRM 381-202. (D) **DPD-1CQ** as detected via SRM 353-231. (E) **DPD-2CQ** as detected via SRM 381-219. (F) Chromatogram **DPD-3CQ** as detected via SRM 409-219.

non-Gaussian peak shape was observed for the quinoxalines resulting from **2** (**DPD-BAQ**). Although **DPD-M1CQ**, the quinoxaline generated from **7**, has only 40% of the peak intensity compared to that of **DPD-EBAQ**, the single molecular species formed in the reaction combined with well-behaved chromatography gave us a promising lead. The esterification greatly enhanced the signals observed, and this phenomenon can be explained by Zwitter ion formation hindering the electrospray ionization process. These tags were originally designed to fly in negative mode, and removing the acid functionality prevents competing ionization.

The reactions of the three candidates with the highest sensitivity, **2**, **3**, and **7**, were next studied via ¹H NMR to characterize the kinetics of quinoxaline formation and gain further structural information. For these experiments, 1 mol equivalent of derivatizing agent was mixed with DPD and observed over 1 h. If the reaction had not reached completion, another mol equivalent was added at 1 h, and if by 2 h the reaction had not

completed, a third equivalent was added. After 2.5 h and 3 equivalents the reaction of DPD and **2** had not reacted to completion, indicating that the reaction was not facile enough for a robust derivatizing agent. Additionally, formation of the two regioisomers was apparent in a 1:1 ratio (Figure 7.4). Formation of **DPD-EBAQ** went to completion in 2 h with only two equivalents. Formation of two regioisomers was still observed in a 1:1 ratio, in spite of the added steric bulk from the ethyl ester. Reaction of 1 equivalent of **7** with DPD led to 90% conversion in 30 min, and addition of a second equivalent at this time yielded 100% conversion within another 30 min. The difference in reaction kinetics can be explained by an increase in amine nucleophilicity on **7**, due to the electron donating ether on the benzene ring, when compared to the electron withdrawing carboxylate in **2** and **3**. The reaction efficiency combined with the production of a single quinoxaline led to the selection of **7** for further validation.

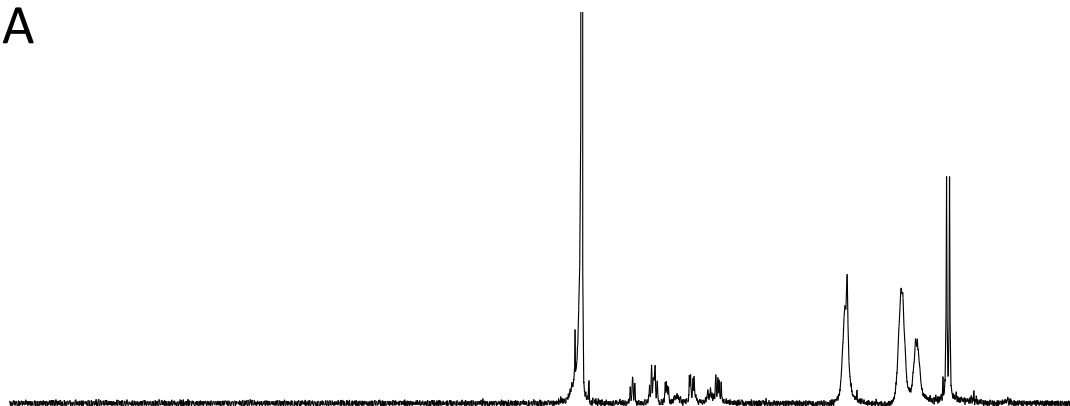
7.5 Assay Linearity and Selectivity

We then addressed the sensitivity, limit of detection and assay linearity using this reagent. Half log serial dilutions of a **DPD-M1CQ** standard (2.35 mM) were made in water and these solutions were analyzed by LC-MS/MS. Each of the four SRMs were monitored, and plots of peak integration in ion counts v. concentration were made for three SRMs (Figure 7.5). The fourth SRM corresponds to a loss of water, and it had a poor signal to noise ratio (S/N). The signal was linear over the entire concentration range and SRM 381-202 had the highest sensitivity, followed closely by 381-231. **DPD-M1CQ** could be detected as low as 230 pM in all SRMs; however, the signal to noise ratio was too low for quantitation at this concentration. The signal to noise ratio at 740 pM was ~5:1 and is sufficient for quantitation. Because the intention was to use relative quantitation using an isotope dilution method, no further calibration or determination of LOD was performed.

The complexity of amine-sugar chemistry in natural systems was an anticipated obstacle to quantification of DPD in complex media. Sugars can undergo several types of reactions in the presence of nucleophilic nitrogen, including Lobry de Bruin-van Ekenstein rearrangements¹⁸⁷ and Maillard reactions.¹⁸⁸ These can lead to the formation of DPD and similar molecules and their respective quinoxalines from other media

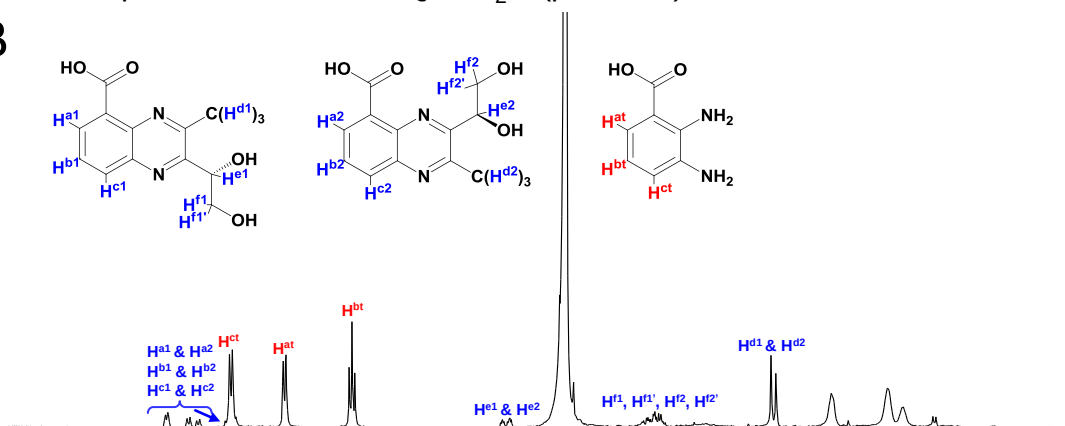
^1H NMR spectrum of **DPD** in D_2O (pD = 1.8)

A



^1H NMR spectrum of **DPD-BAQ** in D_2O (pD = 1.8)

B



^1H NMR spectrum of **DPD-EBAQ** in D_2O (pD = 1.8)

C

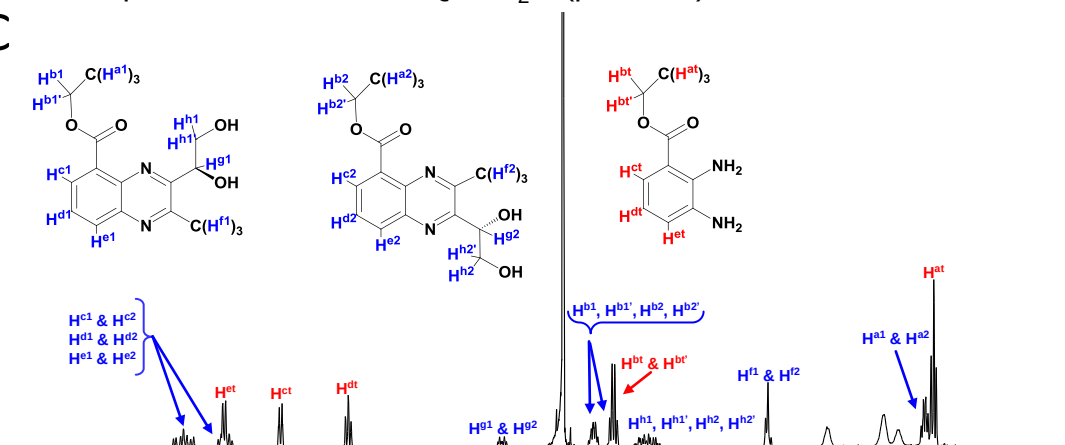


Figure 7.4. ^1H NMR results of structural and kinetic studies

^1H NMR spectrum of **DPD-M1CQ** in D_2O (pD = 1.8)

D

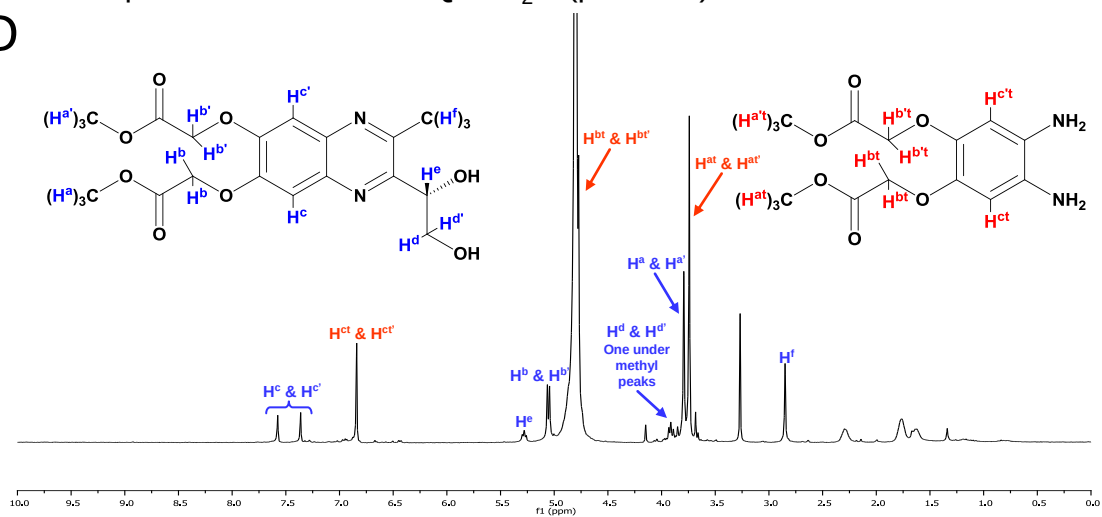


Figure 7.4. Continued.

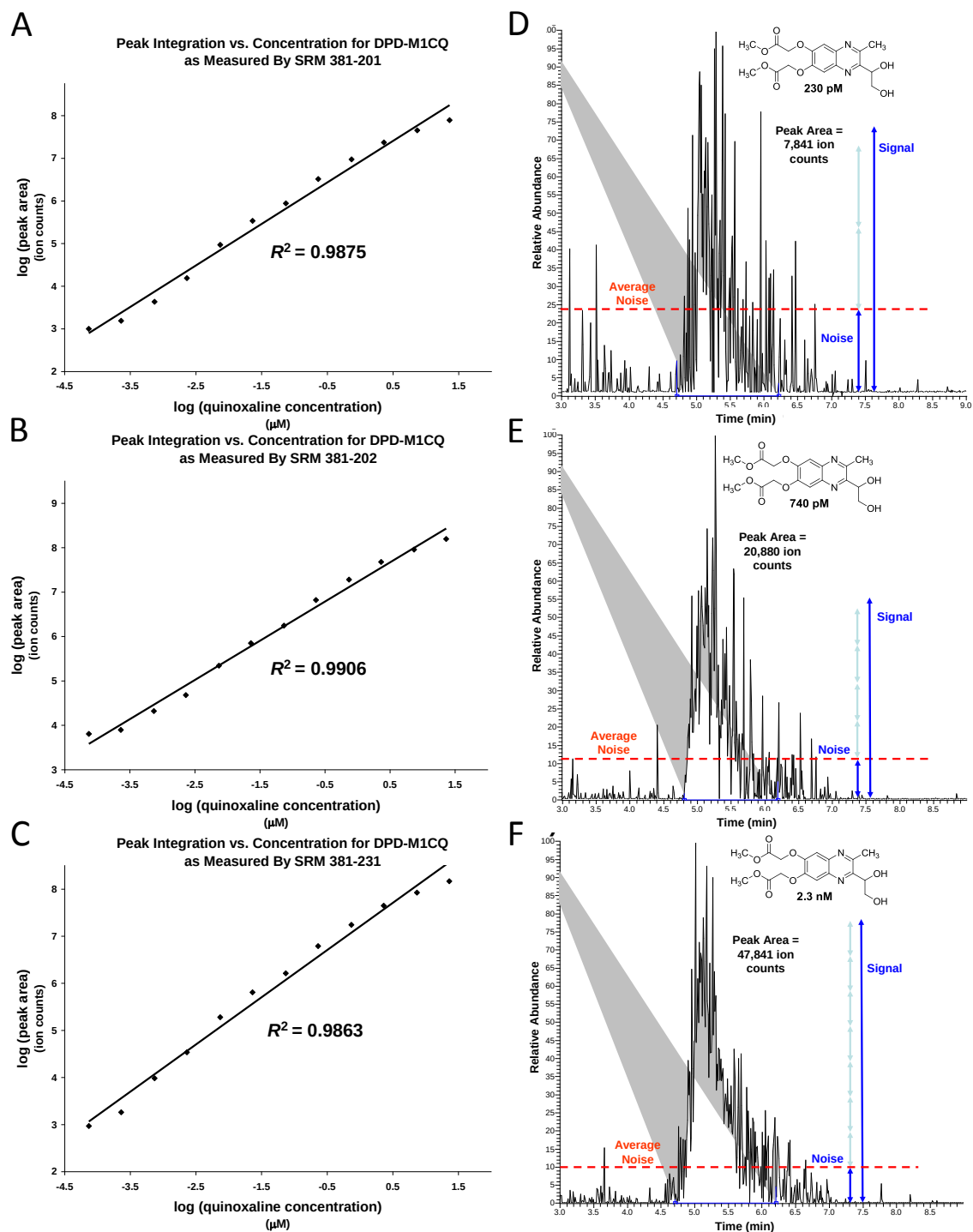
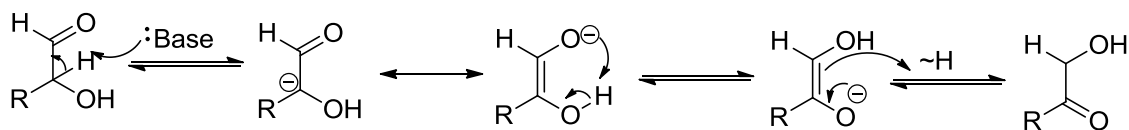


Figure 7.5. Linearity and limit of detection. Calibration curves for **DPD-M1CQ** in water as detected by SRMs (A) 381-201, (B) 381-202, and (C) 381-231. Chromatograms from SRM 381-202 at (D) 230 pM, (E) 740 pM and (F) 2.3 nM.

Lobry-de Bruyn-van Ekenstein Rearrangement



Maillard Reactions

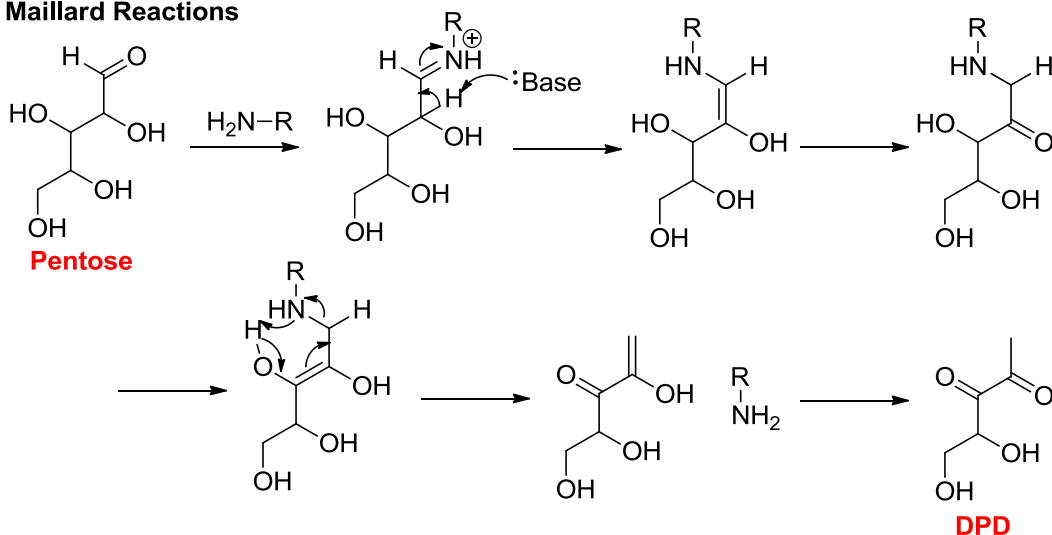


Figure 7.6. Examples of sugar reactions and rearrangements promoted by amines that may lead to false positives in our method.

components (Figure 7.6). These reactions are mostly base promoted, and the majority can be avoided by maintaining a pH below 7.5.¹⁸⁵ We ensured all handling and analysis steps were carried out at a pH 7.2 or below, and to test the selectivity of the conditions, **7** was added to sterile Lysogeny Broth (LB)¹⁸⁹ and incubated 1 h. The samples were then analyzed via LC-MS/MS and monitored for the four SRMs. Comparatively small peaks in three of the SRMs provided evidence of some side reaction with media carbohydrates. However, the 381-202 SRM contained no peak resulting from these side reactions. These samples were reanalyzed after 11 h at 4 °C, and no changes were observed. We therefore believe that quantitation using SRM 381-202 is selective for DPD.

7.6 Introduction of an Internal Standard

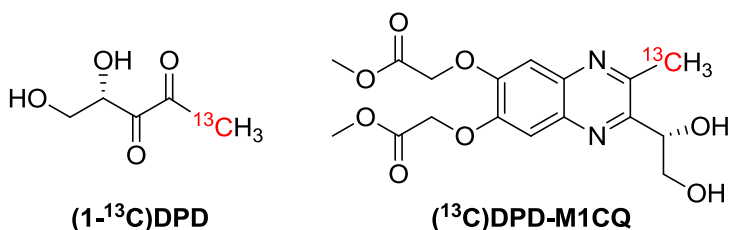
Utilizing a stable isotope labeled internal standard (isotope dilution mass spectrometry) is a powerful mass spectrometry tool for absolute quantitation. Because

the internal standard is chemically identical to the molecule being quantified, this method can account for matrix effects, partitioning, handling errors and the like. There were two choices for the internal standard in this case, addition of a labeled form of our quinoxaline similar to that used for the GC-MS analyses,¹⁸⁵ or addition of a labeled DPD. We anticipated that a quinoxaline may not be best because of differential partitioning or incomplete reaction with DPD. However both options were tested.

First it was necessary to determine the appropriate SRMs for detection to ensure that analogous reactions were being compared. For these experiments the ratio of SRM signals was observed from cell-free supernatants of *E. coli* to which **(1-¹³C)DPD** and **7** had been added (Figure 7.7). The **(1-¹³C)DPD** was synthesized by previously reported methods.¹⁶⁹ For **DPD-M1CQ** in wild type *E. coli* supernatants, a ratio of 22:43:47:100 was consistently observed for SRMS 381-201:381-202:381-231:381-363, respectively. For **(¹³C)DPD-M1CQ** a total of six SRMs could be analogous to the four established for **DPD-M1CQ**. If the ¹³C-isotope remained in the fragment, SRMs 382-202, 382-203, 382-232, and 382-364 would be analogous, and if the isotope was lost in the fragmentation reaction, SRMs 382-201, 382-202*, and 382-231 would be analogous. There is possible overlap with the 382-202 SRM and 382-364 is a loss of water and cannot therefore lose the carbon of interest. When the ratio of these six SRMs were measured in cell-free supernatants of *luxS⁻ E. coli*, a ratio of 1:22:43:41:100 was measured for 382-201:382-202:382-203:382-232:382-364, respectively. This is very similar to the ratio observed for **DPD-M1CQ** and we can therefore conclude that the methyl group is retained during fragmentation, and SRMs 381-202 and 382-203 represent analogous fragmentation reactions.

We then tested **(1-¹³C)DPD** and **(¹³C)DPD-M1CQ** for utility as an internal standard. Each was added to aliquots of a *V. harveyi* BB120 culture grown in Luria-Marine Medium¹⁵³ for 8.5 h at a final concentration of 10 μM. Excess **7** (130 equivalents) was then added and the reaction was allowed to proceed for 1 h. Analysis of these samples via LC-MS/MS was followed

by calculation of [DPD] by determining the signal ratios from SRMs 381-202 and 382-203 (corrected as described



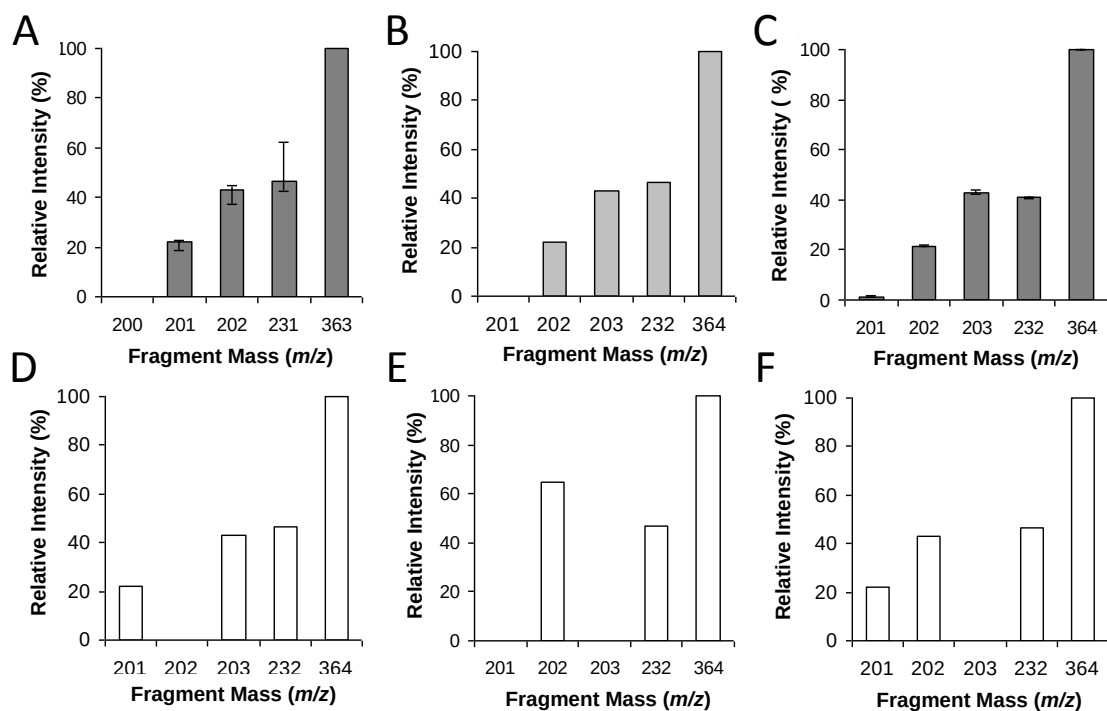


Figure 7.7. Ratios of SRM intensities for **DPD-M1CQ** and **(¹³C)DPD-M1CQ**. The intensity of either SRM 381-363 or 382-364 was normalized to 100% for each sample, and the signal for the other SRMs are reported as a percent of the relative intensity of that peak. (A) Measured ratio of the intensities for the most sensitive SRMs used for **DPD-M1CQ** concentration determination. (B) Expected ratio of intensities for the SRMs needed to detect **(¹³C)DPD-M1CQ** if the ¹³C-methyl remains in the fragment. (C) Measured ratio of the SRM intensities found during **(¹³C)DPD-M1CQ** concentration determination. (D) Expected ratio of intensities for **(¹³C)DPD-M1CQ** SRMs if fragment 202 has lost the ¹³C-label. (E) Expected ratio of intensities for **(¹³C)DPD-M1CQ** SRMs if fragment 203 has lost the ¹³C-label. (F) Expected ratio of intensities for **(¹³C)DPD-M1CQ** SRMs if both fragment 202 and 203 have lost the ¹³C-label.

below) and multiplying by the added internal standard concentration (Equation 7.1). The quinoxaline internal standard measured the [DPD] in these samples to be 5.6 μM and the DPD internal standard measured the [DPD] at 12.3 μM . The quinoxaline underestimated the concentration by more than half, probably due to differential partitioning. An incomplete reaction at 130 equivalents of excess **7** is less likely. These experiments confirmed that (1- ^{13}C)DPD standard was a better option moving forward.

Equation 7.1
$$[\text{DPD}] = \frac{\text{signal } 381-202}{\text{signal } 382-203} * \text{known } [(1-^{13}\text{C})\text{DPD}]$$

A (1- ^{13}C)DPD internal standard is not ideal for isotope dilution mass spectrometry because a mass difference of only 1 m/z will overlap with the $[\text{M}+1+\text{H}]^+$ peak. Since neither of the two reported DPD syntheses allow for the facile introduction of additional isotopes, we developed a correction method. An SRM method decreases the contribution from naturally occurring isotopes because the isotope must be included in the parent and fragment ion to be observed. When a **DPD-M1CQ** standard was measured in water, the signal in SRM 382-203 was measured to be 14.4% of the naturally occurring $[\text{M}+\text{H}]^+$ peak in 381-202. Another source of error can arise from the small percentage of the ^{13}C -methyl iodide used in the (1- ^{13}C)DPD synthesis that is ^{12}C , so a second correction factor was introduced. Measurements of (^{13}C)DPD-M1CQ in water detected a peak in SRM 381-202 that was 0.8% of the signal in SRM 382-203. These sources of error become more significant at certain ratios of DPD to internal standard. In particular, when the DPD signal is 30% of the internal standard signal, the natural abundance of isotopes becomes a contributing factor, but when it is below 20% of the internal standard signal, the methyl iodide impurity becomes more of a factor. Between these two signal ratios, the two correction factors balance each other out (Table A7.1).

7.7 *In vivo* Validation and Application

To validate our method *in vivo*, extracellular DPD production was measured in the cell-free supernatants of wild type and *luxS*⁻ strains of two model species, *V. harveyi* and *E. coli*. These two species were chosen because DPD production for both has been studied by other methods. Overnight cultures were inoculated 1:50 into fresh media and

the cultures were regularly sampled into early stationary phase. The samples were spiked with internal standard to a final concentration of 10 μM , and then centrifuged to remove the cells. Afterwards, **7** was added to a final concentration of 1.3 mM and samples were allowed to react for 1 h before LC-MS/MS analysis (Figure 7.8).

Production of DPD by *V. harveyi* was observed to gradually increase through logarithmic growth and then drop off gradually after reaching stationary phase. The highest concentration measured was 22 μM at 6.5 h in the wild type strain BB120. No DPD was measured in the *luxS*⁻ strain MM30. These experiments were completed in duplicate on two separate days for a total of four measurements. Triplicate injections of the same sample had lower variability than both the inter- and intra-day variability (Table A7.2).

E. coli production of DPD was observed to increase overtime until 3.5 h when the signal dropped steeply. This sharp decrease in extracellular [DPD] was likely due to the induction of the LsrB transporter which imports DPD for degradation. The peak concentration in the wild type (BW25113) was 21 μM . No DPD was observed in the *luxS*⁻ cultures. This experiment was completed in duplicate in one day, and on a second day several time points were tested although not repeated in full.

These results demonstrate that our method is not simply qualitatively useful for detecting differences between strains capable of DPD synthesis and those that are not, or for the detection of DPD uptake. The method is also quantitatively useful since our measurements found [DPD] to be in the μM range for both species, as previously reported.

After successfully validating this method *in vivo*, saliva was chosen as a clinically relevant complex medium to extend the breadth of the method's application. DPD is known to be a signal in the oral cavity by mediating establishment of mutual biofilms of *S. oralis* and *A. naesalundi*.¹⁸⁶ We sought to determine whether DPD could be measured from human saliva and for this purpose enlisted fifteen volunteer donors. A saliva sample from each volunteer was spiked with 1 μM (1-¹³C)DPD and centrifuged to remove any particulates. Supernatants were mixed with **7** and allowed to react 1 h prior to LC-MS/MS analysis. Each sample was analyzed twice, and [DPD] was variable between individuals ranging from 287 to 1096 nM (Figure 7.9).

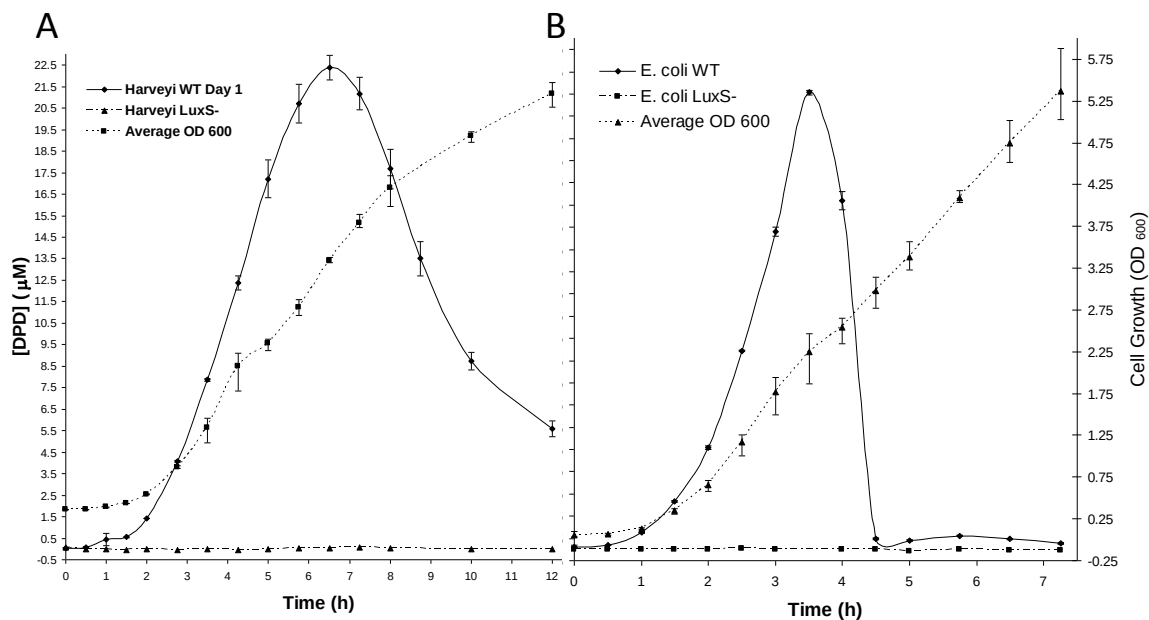


Figure 7.8. Production of DPD in relation to cell growth during the exponential growth phase. A) Autoinducer production by *V. harveyi*. B) Autoinducer production by *E. coli*. Measurement of the DPD concentration for each WT species was performed in duplicate and the average data are reported. Although experiments for the *luxS*⁻ strains were performed in duplicate, [DPD] measurement for every time point was only performed once. For select time points, measurement was conducted in duplicate to confirm that no DPD was detected in the *luxS*⁻ strains. The average OD₆₀₀ for all four cultures, two WT and two *luxS*⁻, was used as no growth differences were observed between the strains. Error bars in the graphs indicate the range of the data.

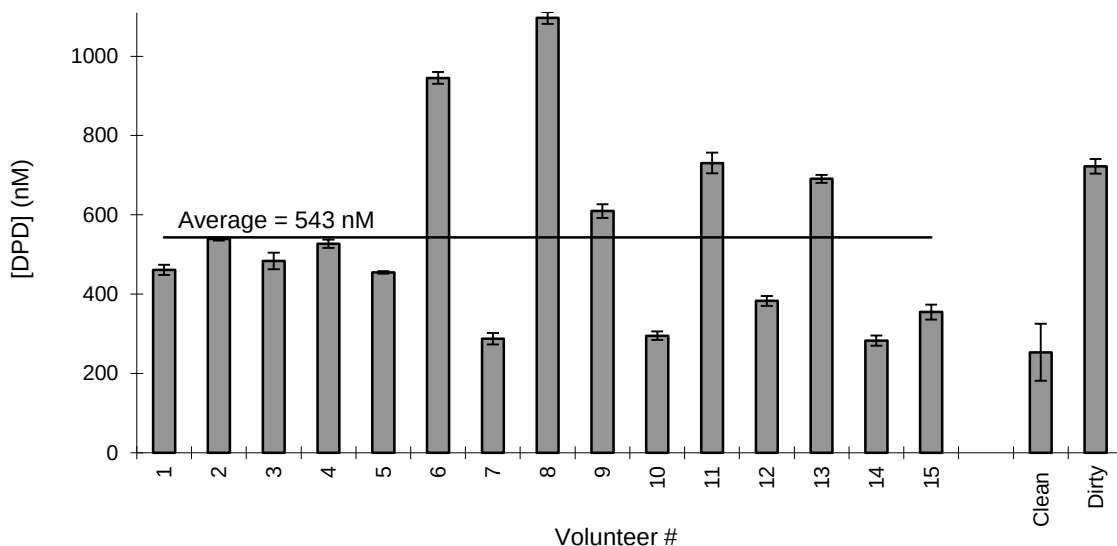


Figure 7.9. Bacteria in the human oral cavity produce **DPD** in sufficient concentrations for detection. Human Saliva was collected, and tag **7** was added to derivatize **DPD** for detection. All volunteers had a measurable [DPD] in their saliva with an average concentration of 543 nM. The sample from each volunteer was analyzed in duplicate, and the chart displays the average of these measurements.

The average concentration was 543 nM, which is about 2x the concentration measured in model studies of biofilm formation in sterile saliva.¹⁸⁶ Two of the volunteers had much higher concentrations of DPD than the others, however this phenomenon is not yet understood. This result prompted one more experiment in which the saliva of volunteer 13 was sampled after the oral biofilm was allowed to grow for 24 h (dirty), and after an oral hygiene regimen including brushing, flossing and swishing mouth wash (clean). A clear distinction in saliva [DPD] could be made between the clean (253 nM) and dirty (722 nM) samples. Note that the concentrations in the dirty sample were consistent with the concentration measured on a different day.

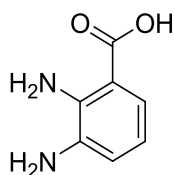
7.8 Conclusions

Lack of a quantitative method for detecting DPD has left open questions about the relevance of AI-2 *in vivo*. Our detection method has several advantages over previously reported assays. First it is a direct chemical measurement, which avoids many of the

problems introduced by variability in biological systems and facilitates measurement in complex systems. We have improved the derivatizing agent itself by addition of electron donating groups, which increase the rate of reaction with DPD and the number of quinoxalines protonated at a given time. The ether tails also fragment easily and selectively allowing more sensitive and selective measurement of DPD. More improvements are needed due to the length of each chromatographic run, and current research aims to build upon the work reported in this chapter by development of a more high throughput method. A four min chromatographic method has already been developed, and work on a colorimetric or fluorescent based assay is underway.

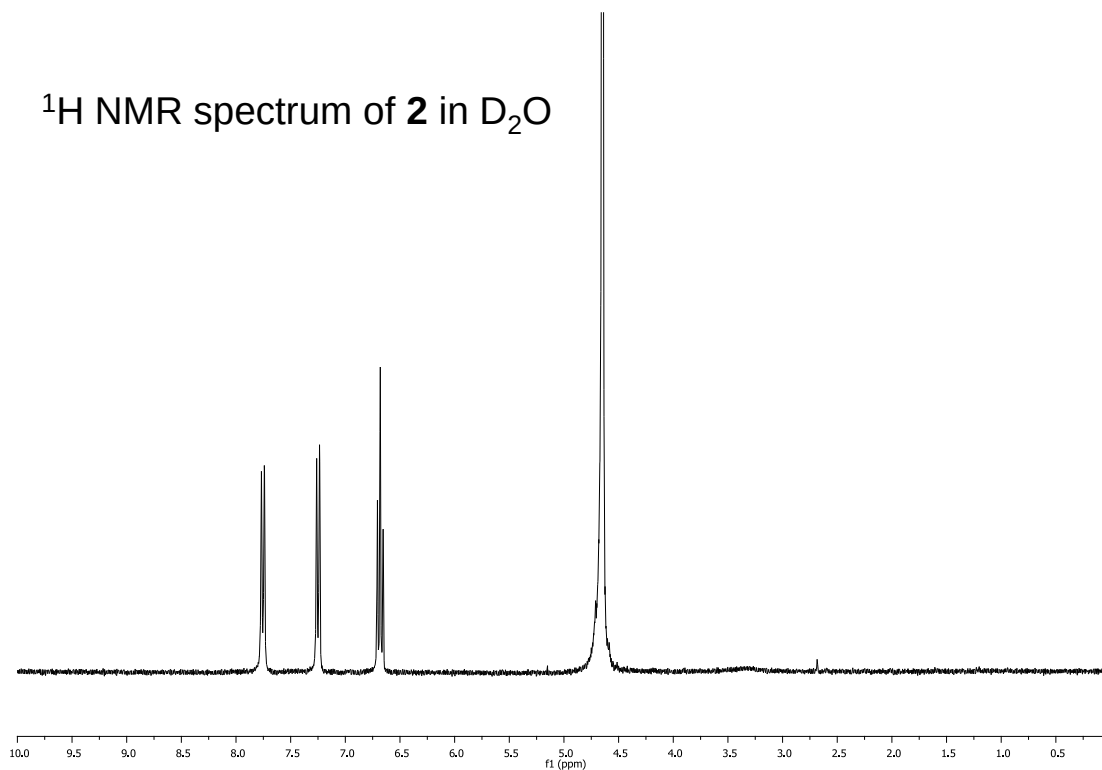
Using these methods we have reported the first direct chemical quantitation of AI-2 from complex, clinically relevant media. Our *in vivo* experiments successfully reproduced earlier results in both *V. harveyi* and *E. coli*.

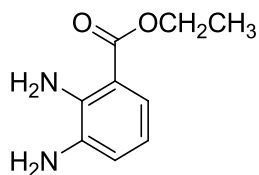
7.8 Methods



2

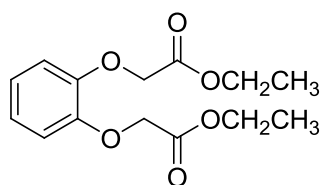
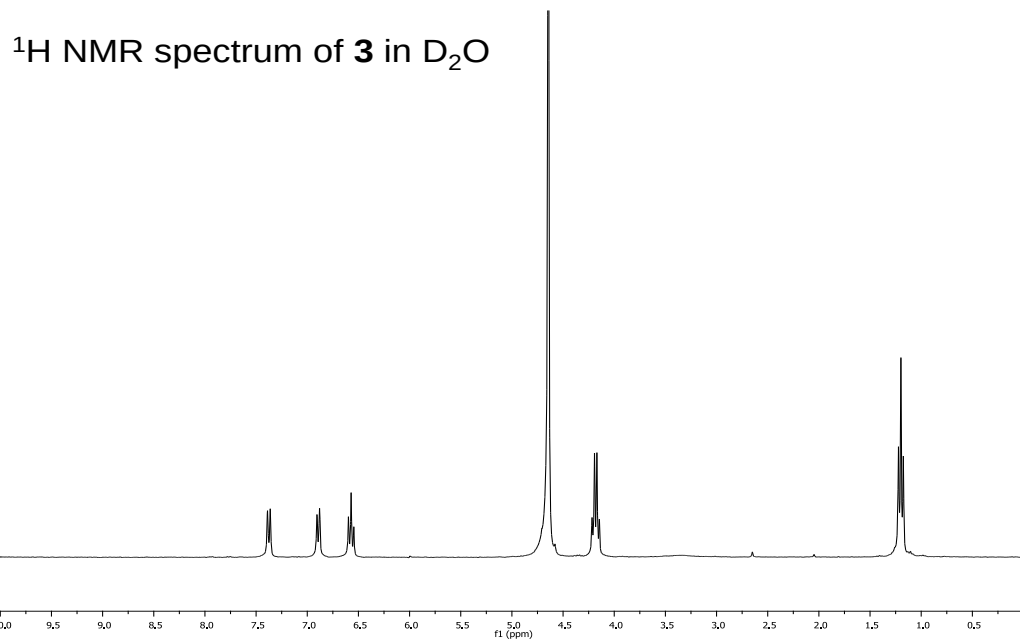
2,3-Diaminobenzoic acid, 2. The known compound **2** was synthesized following a previous procedure¹⁹⁰ with only a slight modification to the solvent used. A solution of 2-amino-3-nitro-benzoic acid (0.9609 g, 5.28 mmol) in ethanol (56 mL) was hydrogenated in a PARR apparatus using 5% Pd/C (0.7011 g, 6.59 mmol) catalyst at 35 bar H₂ for 1.5 h. The reaction was then filtered and concentrated *in vacuo* to give **2** as an impure solid which was then redissolved in water (30 mL) and acidified to pH 2 using concentrated HCl. The resulting mixture was filtered through celite, and the filtrate was concentrated *in vacuo* to give pure **2** as a red solid. ¹H NMR (300 MHz, D₂O) δ 7.75 (d, J = 8.1 Hz, 1H), 7.25 (d, J = 7.8 Hz, 1H), 6.68 (t, J = 7.9 Hz, 1H).





3

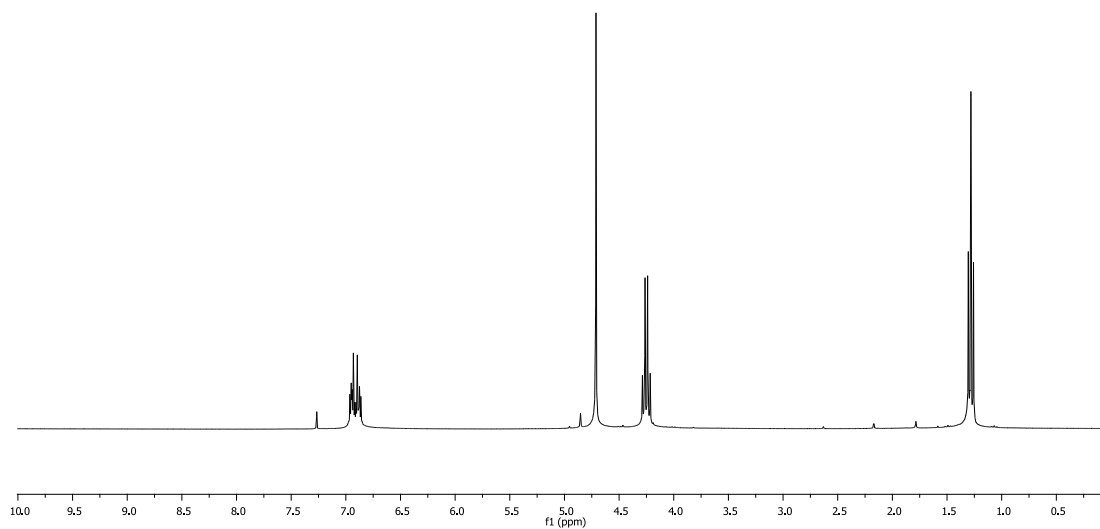
Ethyl 2,3-diaminobenzoate, 3. Synthesis of known compound **3**^{191,192} was performed by the modified procedure below. A solution of **2** (0.482 g, 3.17 mmol) and 95% ethanol (40 mL) in a pressure vessel was cooled to 0 °C. Thionyl chloride (2.4 mL, 32.9 mmol) was then added dropwise. The reaction vessel was then sealed, and the reaction was heated with stirring to 100 °C for 24 h. At this time, the reaction was cooled to room temperature and extracted with dichloromethane (3 x 20 mL). The resulting organic layers were combined and dried with MgSO₄. Following the removal of the MgSO₄, the dried organic layer was concentrated *in vacuo* to give a dark brown/black oil. This oil was then redissolved in dichloromethane (10 mL) and added to a pad of silica on a filtration apparatus. After elution of impurities with a 7:3 v/v hexanes:ethyl acetate (50 mL), the product is recovered via elution with 100% ethyl acetate. A yellow band of product is noted during this process. Concentration *in vacuo* yields clean **3** as a yellowish oil. (0.249 g, 1.382 mmol, 43.6%). ¹H NMR (300 MHz, D₂O) δ 7.37 (d, J = 8.1 Hz, 1H), 6.89 (d, J = 7.5 Hz, 1H), 6.57 (t, J = 7.9 Hz, 1H), 4.18 (q, J = 7.1 Hz, 2H), 1.20 (t, J = 7.1 Hz, 3H).

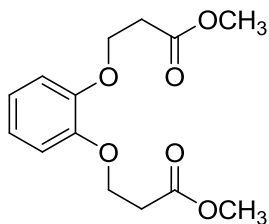


8a

Diethyl 2,2'-(1,2-phenylenebis(oxy))diacetate, 8a. Known compound **8a** was synthesized via slight modification of a previously reported procedure.¹⁹³ Under inert atmosphere, potassium carbonate (25.048 g, 182 mmol) was added to a solution of catechol (5.030 g, 45.4 mmol) in dry acetone (100 mL). Ethyl bromoacetate (9.97 mL, 90 mmol) was then added to the suspension, and the mixture was refluxed for 48 h. The excess carbonate was removed by filtration, and the filtrate was concentrated *in vacuo* to give a brown oil. This oil was then dissolved in diethyl ether (40 mL). The resulting solution was then washed with 5% sodium hydroxide in water (80 mL) and brine (2 x 80 mL), and the organic layer was dried with MgSO_4 and concentrated *in vacuo* to give 6.222 g (48.5%) crude **8a** as a light brown oil which was used in subsequent reactions without further purification. ^1H NMR (300 MHz, CDCl_3) δ 6.91 (m, 4H), 4.25 (q, J = 7.1 Hz, 4H), 1.28 (t, J = 7.1 Hz, 6H).

^1H NMR spectrum of **8a** in CDCl_3

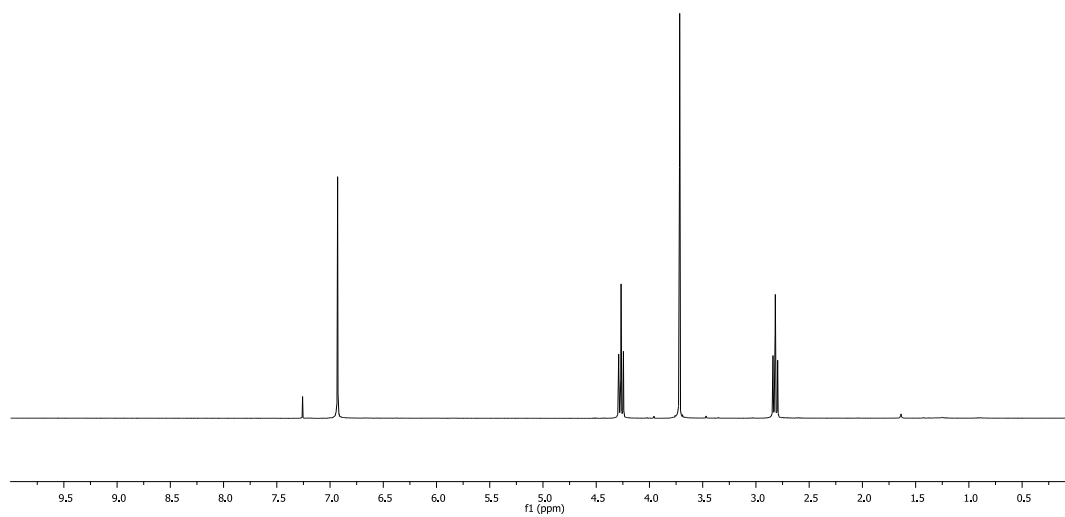


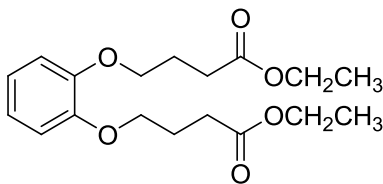


8b

Dimethyl 3,3'-(1,2-phenylenebis(oxy))dipropionate, 8b. Catechol (10.00 g, 91.00 mmol) was placed in a pressure vessel and completely dissolved in methyl acrylate (32.70 mL, 363.00 mmol). Sodium methoxide (0.49 g, 9.08 mmol) was then added to the solution, and shaking was continued until all solid was dissolved. The reaction was then sealed, stirred, and heated to 100° C for 24 h. Methyl acrylate was removed via distillation *in vacuo* yielding a brown residue which was then dissolved in diethyl ether (40 mL). The resulting solution was washed with 5% sodium hydroxide in water (40 mL) and brine (2 x 40 mL). The organic layer was then dried with MgSO₄ and concentrated *in vacuo* to give **8b** as a pale white, oily solid (1.21 g, 4.29 mmol, 4.72%). mp: 43 °C; ¹H NMR (300 MHz, CDCl₃) δ 6.93 (s, 4H), 4.27 (t, J = 6.6 Hz, 4H), 3.72 (s, 6H), 2.82 (t, J = 6.6 Hz, 4H); ¹³C NMR (75 MHz, CDCl₃) δ 171.76, 148.96, 122.27, 115.63, 65.29, 52.06, 34.73; HRMS-DART (*m/z*): [M+H]⁺ calculated for C₁₄H₁₉O₆ 283.11816; found, 283.11879.

^1H NMR spectrum of **8b** in CDCl_3

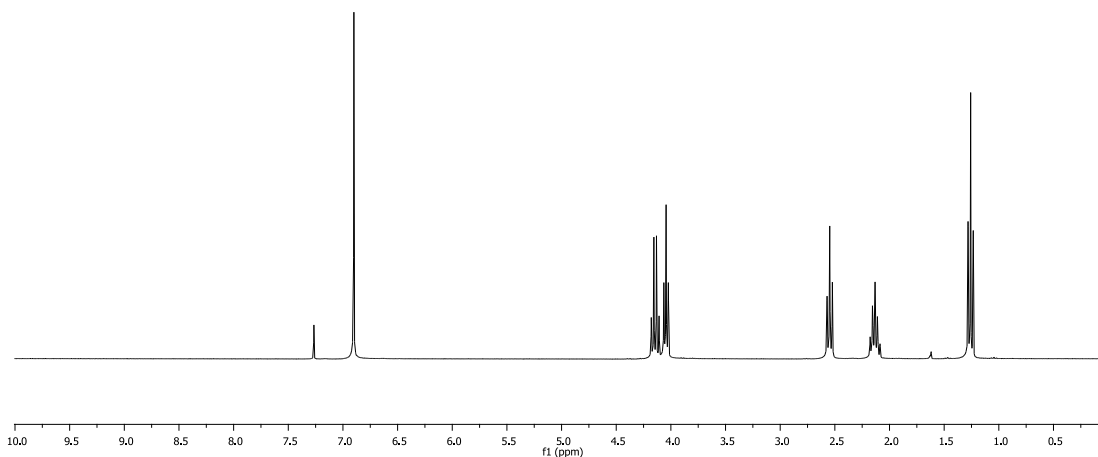


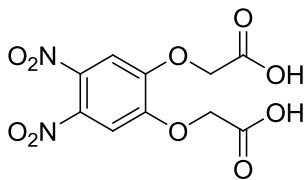


8c

Diethyl 4,4'-(1,2-phenylenebis(oxy))dibutanoate, 8c. Known compound **8c** was synthesized according to a previously reported literature procedure.¹⁹⁴ Briefly, potassium carbonate (25.224 g, 182 mmol) was added to a solution of catechol (5.056 g, 45.4 mmol) in dry acetone (100 mL) under inert atmosphere. Ethyl 4-bromobutyrates (12.87 mL, 90 mmol) was then added to the suspension and the reaction was refluxed for 48 h. The excess carbonate was removed by filtration, and the filtrate was concentrated *in vacuo* to give a brown oil. This oil was then dissolved in diethyl ether (40 mL). After washing with 5% sodium hydroxide in water (80 mL) and brine (2 x 80 mL), the organic layer was dried with MgSO₄ and concentrated *in vacuo* to give 12.169 g (79%) crude **8c** as a light brown oil which was used in subsequent reactions without further purification. ¹H NMR (300 MHz, CDCl₃) δ 6.90 (s, 4H), 4.14 (q, J = 7.1 Hz, 4H), 4.04 (t, J = 6.2 Hz, 4H), 2.54 (t, J = 7.3 Hz, 4H), 2.13 (m, 4H), 1.26 (dd, J = 9.3 Hz, 4.9 Hz, 6H).

¹H NMR spectrum of **8c** in CDCl₃

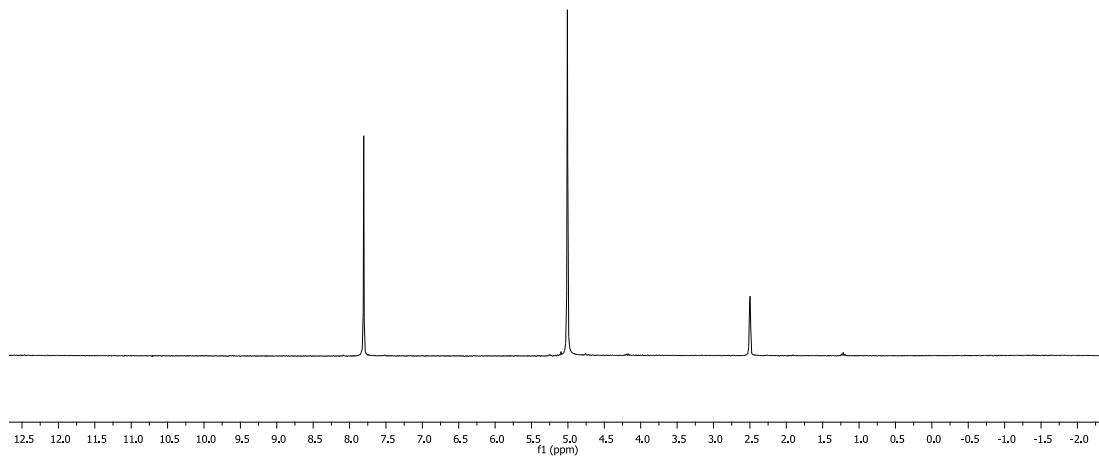


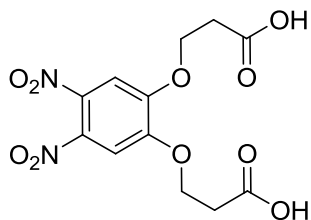


9a

2,2'-(4,5-dinitro-1,2-phenylene)bis(oxy)diacetic acid, 9a. Concentrated HNO₃ (4 mL) was added dropwise to a solution of **8a** (0.685 g, 2.427 mmol) in sulfuric acid (4 mL) at 0 °C. The reaction flask was then sealed, and the reaction was stirred at room temperature. After 24 h a precipitate had formed, and water (4 mL) was added dropwise to the reaction. The reaction was frozen and thawed to facilitate precipitation of a white solid which was then collected via filtration and washed with cold water (3 x 25 mL) to yield **9a** as a white solid (0.488 g, 1.543 mmol, 63.6%). mp: 182 °C; ¹H NMR (300 MHz, DMSO-*d*₆) δ 7.79 (s, 2H), 4.99 (s, 4H); ¹³C NMR (75 MHz, DMSO-*d*₆) δ 169.93, 150.52, 136.39, 110.19, 66.29; HRMS-DART (*m/z*): [M-H]⁻ calculated for C₁₀H₇N₂O₁₀ 315.01007; found, 315.01348.

¹H NMR spectrum of **9a** in DMSO-*d*₆

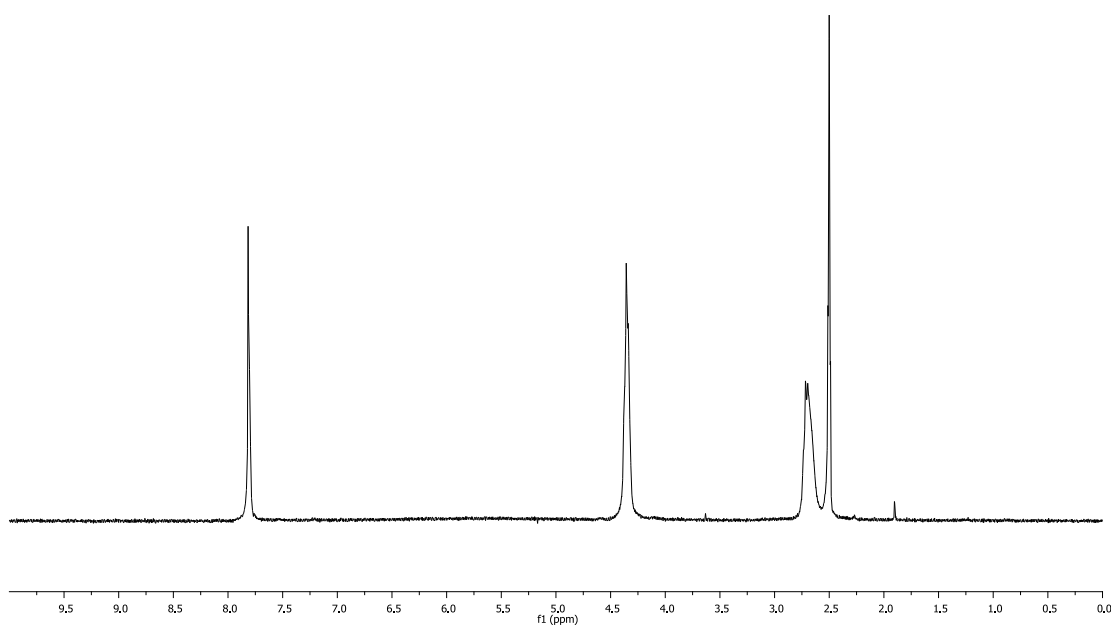


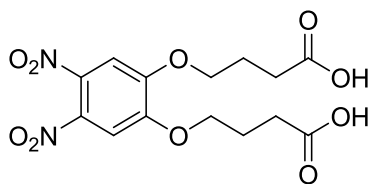


9b

3,3'-(4,5-dinitro-1,2-phenylene)bis(oxy)dipropionic acid, 9b. Water (198 μL) was added to concentrated HNO_3 (6.5 mL) and the resulting solution was added dropwise to a solution of **8b** (0.517 g, 1.831 mmol) in acetic acid (6.5 mL) at 0 °C. The reaction flask was then sealed and stirred at 50° C for 11 h. At this time, water (6.7 mL) was added, and the reaction was allowed to stir for 1.5 h. The reaction was then frozen and thawed to facilitate precipitation of a yellow solid. This precipitate was collected via filtration and washed with cold water (3 x 30 mL) to yield **9b** as a yellow solid (0.375 g, 1.089 mmol, 59.5%). mp: 155 °C; ^1H NMR (300 MHz, $\text{DMSO}-d_6$) δ 7.82 (s, 2H), 4.37 (t, J = 5.9 Hz, 4H), 2.75 (t, J = 5.9 Hz, 4H); ^{13}C NMR (75 MHz, $\text{DMSO}-d_6$) δ 172.24, 151.12, 136.64, 109.86, 66.61, 34.39; HRMS-DART (m/z): $[\text{M}-\text{H}]^-$ calculated for $\text{C}_{12}\text{H}_{11}\text{N}_2\text{O}_{10}$ 343.04137; found, 343.03795.

^1H NMR spectrum of **9b** in $\text{DMSO}-d_6$

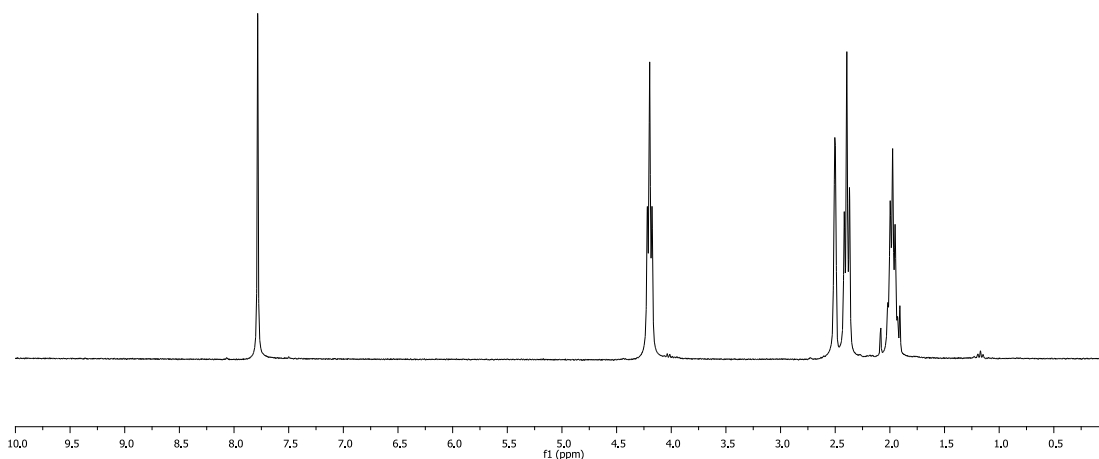


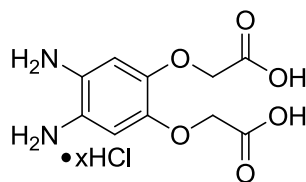


9c

4,4'-(4,5-dinitro-1,2-phenylene)bis(oxy)dibutanoic acid, 9c. Water (121 μL) was added to concentrated HNO_3 (4.0 mL) and the resulting solution was added dropwise to a solution of **8c** (0.318 g, 0.940 mmol) in acetic acid (4.0 mL) at 0 $^\circ\text{C}$. The reaction flask was then sealed and stirred at 50 $^\circ\text{C}$ for 11 h. Water (4.0 mL) was then added, and the reaction was stirred for 1.5 h. At this time, the reaction was frozen and thawed to facilitate precipitation of a yellow solid. This precipitate was collected via filtration and was washed with cold water (3 x 25 mL) to yield **9c** as a yellow solid (0.335 g, 0.900 mmol, 96.0%). mp: 162.3 $^\circ\text{C}$; ^1H NMR (300 MHz, $\text{DMSO}-d_6$) δ 12.50 (s, 2H), 7.83 (s, 2H), 4.36 (t, $J = 6.1$ Hz, 4H), 2.75 (t, $J = 6.1$ Hz, 4H); ^{13}C NMR (101 MHz, $\text{DMSO}-d_6$) δ 174.37, 151.65, 136.37, 109.30, 69.41, 30.19, 24.25; HRMS-DART (m/z): $[\text{M}-\text{H}]^-$ calculated for $\text{C}_{14}\text{H}_{15}\text{N}_2\text{O}_{10}$ 371.07267; found, 371.07083.

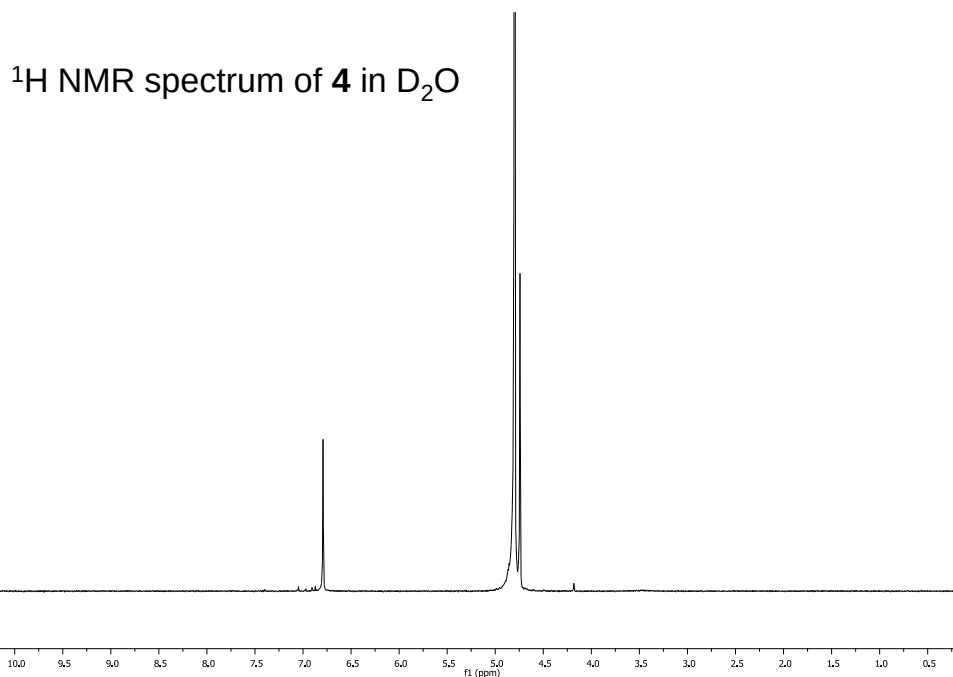
^1H NMR spectrum of **9c** in $\text{DMSO}-d_6$

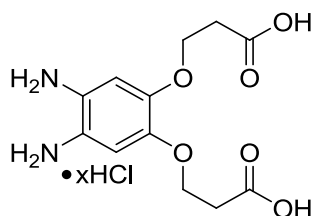




4

2,2'-(4,5-diamino-1,2-phenylene)bis(oxy)diacetic acid • xHCl, 4. To a solution of tin (II) chloride (1.905 g, 8.44 mmol) in concentrated HCl (20 mL) was added **9a** (0.402 g, 1.271 mmol) in one portion. The reaction was then heated to 50 °C and stirred in air for 2.5 h. Concentration of the reaction *in vacuo* gave a brown oil that was then coevaporated with methanol (3 x 30 mL) to give a brown solid. The resulting solid was dissolved in water (50 mL), and hydrogen sulfide was bubbled through the solution to precipitate the tin as an insoluble brown sulfide. The mixture was then filtered through celite with water (2 x 15 mL), and the filtrate was collected. Further treatment of the filtrate with hydrogen sulfide followed by another filtration through celite was performed to insure complete removal of tin from the product. Concentration of the solution *in vacuo* yielded the hydrochloride salt, **4**, as a purple solid (0.421 g, 1.280 mmol, quantitative yield). mp: 195 °C dec; ^1H NMR (300 MHz, D_2O) δ 6.64 (s, 2H), 4.58 (s, 4H); ^{13}C NMR (101 MHz, D_2O) δ 173.03, 144.56, 122.61, 108.75, 66.43; HRMS-DART (m/z): $[\text{M}+\text{H}]^+$ calculated for $\text{C}_{10}\text{H}_{13}\text{N}_2\text{O}_6$ 257.07736; found, 257.07842.

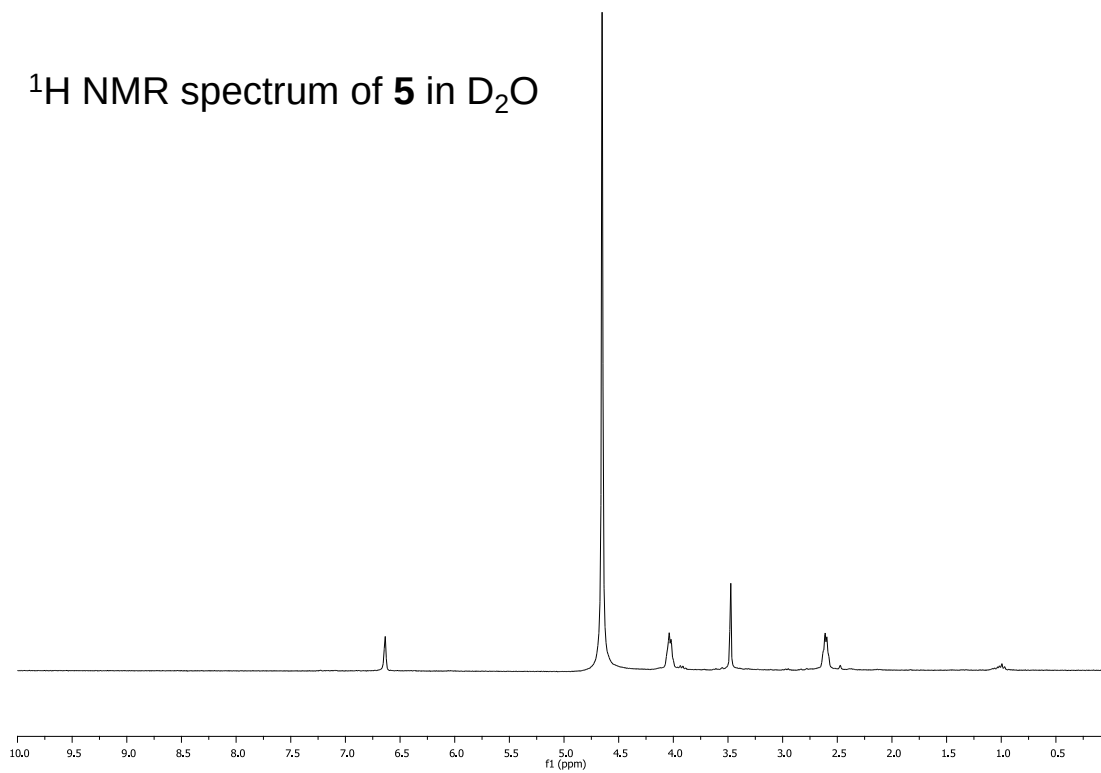


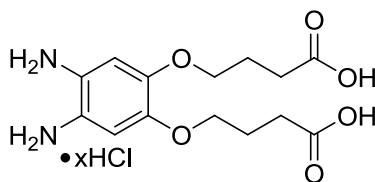


5

3,3'-(4,5-diamino-1,2-phenylene)bis(oxy)dipropionic acid• xHCl, 5. To a solution of tin (II) chloride (83 mg, 0.370 mmol) in concentrated HCl (1 mL) was added **9b** (19.5 mg, 0.057 mmol) in one portion. The reaction mixture was then heated to 50 °C and stirred in air for 2.5 h. Concentration of the solution *in vacuo* gave a brown oil that was then coevaporated with methanol (3 x 5 mL) to give a brown solid. The resulting solid was dissolved in water (30 mL), and hydrogen sulfide was bubbled through the solution to precipitate the tin as an insoluble brown sulfide. The mixture was then filtered through celite with water (2 x 15 mL), and the filtrate was collected. Further treatment of the filtrate with hydrogen sulfide followed by another filtration through celite was performed to insure complete removal of the tin from the product. Concentration of the solution *in vacuo* yielded the hydrochloride salt, **5**, as a purple solid (10.2 mg, 0.029 mmol, 50.4%). mp: > 230 °C; ¹H NMR (300 MHz, D₂O) δ 6.83 (s, 2H), 4.05 (t, J = 5.9 Hz, 4H), 3.64 (s, partial exchange with solvent), 2.54 (t, J = 7.0 Hz, 4H), 2.05 (s, 4H); ¹³C NMR (101 MHz, CD₃OD) δ 171.60, 147.96, 119.80, 110.08, 65.73, 33.66; MS-DART (*m/z*): [2M + Sn]²⁺ was observed. The molecular ion for **5** was elusive. However, the mass of the corresponding Sn compound was found to be 339 *m/z*.

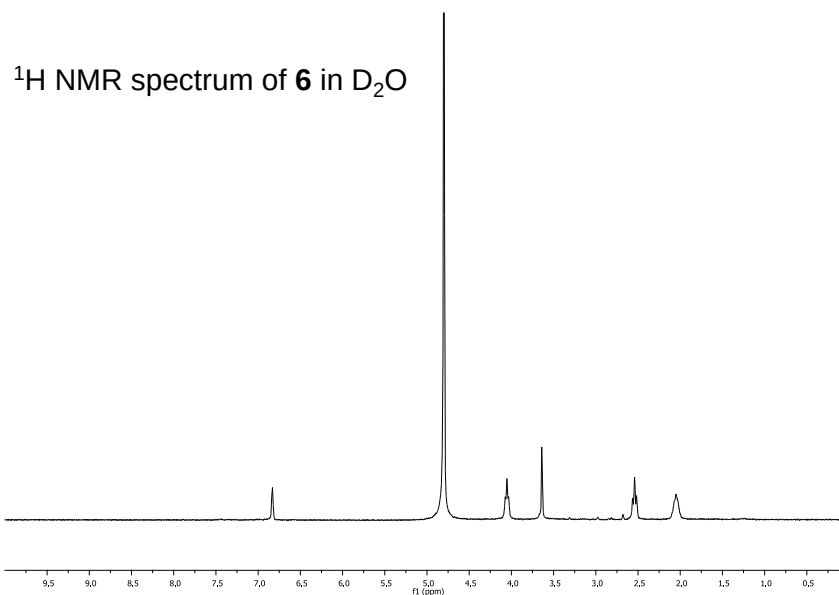
^1H NMR spectrum of **5** in D_2O

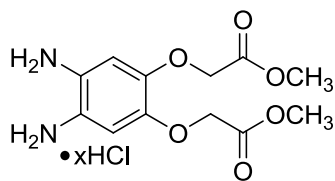




6

4,4'-(4,5-diamino-1,2-phenylene)bis(oxy)dibutanoic acid • xHCl, 6. To a solution of tin (II) chloride (0.108 g, 0.478 mmol) in concentrated HCl (3 mL) was added **9c** (0.028 g, 0.072 mmol) in one portion. The reaction was then heated to 50 °C and stirred in air for 2.5 h. Concentration of the reaction *in vacuo* gave a brown oil that was then coevaporated with methanol (3 x 10 mL) to yield a brown solid. The resulting solid was dissolved in water (20 mL), and hydrogen sulfide was bubbled through the solution to precipitate the tin as an insoluble brown sulfide. The mixture was then filtered through celite with water (2 x 15 mL), and the filtrate was collected. Further treatment of the filtrate with hydrogen sulfide followed by another filtration through celite was performed to insure complete removal of the tin from the product. Concentration of the solution *in vacuo* yielded the hydrochloride salt, **6**, as a purple solid (0.011 g, 0.030 mmol, 40.8%). mp: > 230 °C; ^1H NMR (300 MHz, D_2O) δ 6.81 (s, 2H), 4.24 (m, 4H), 3.68 (s, partial exchange with solvent), 2.76 (m, 4H); ^{13}C NMR (101 MHz, CD_3OD) δ 173.92, 148.37, 119.05, 109.31, 68.84, 29.89, 24.14; HRMS-DART (m/z): $[\text{M}+\text{H}]^+$ calculated for $\text{C}_{14}\text{H}_{20}\text{N}_2\text{O}_6$ 313.13996; found, 313.13888.

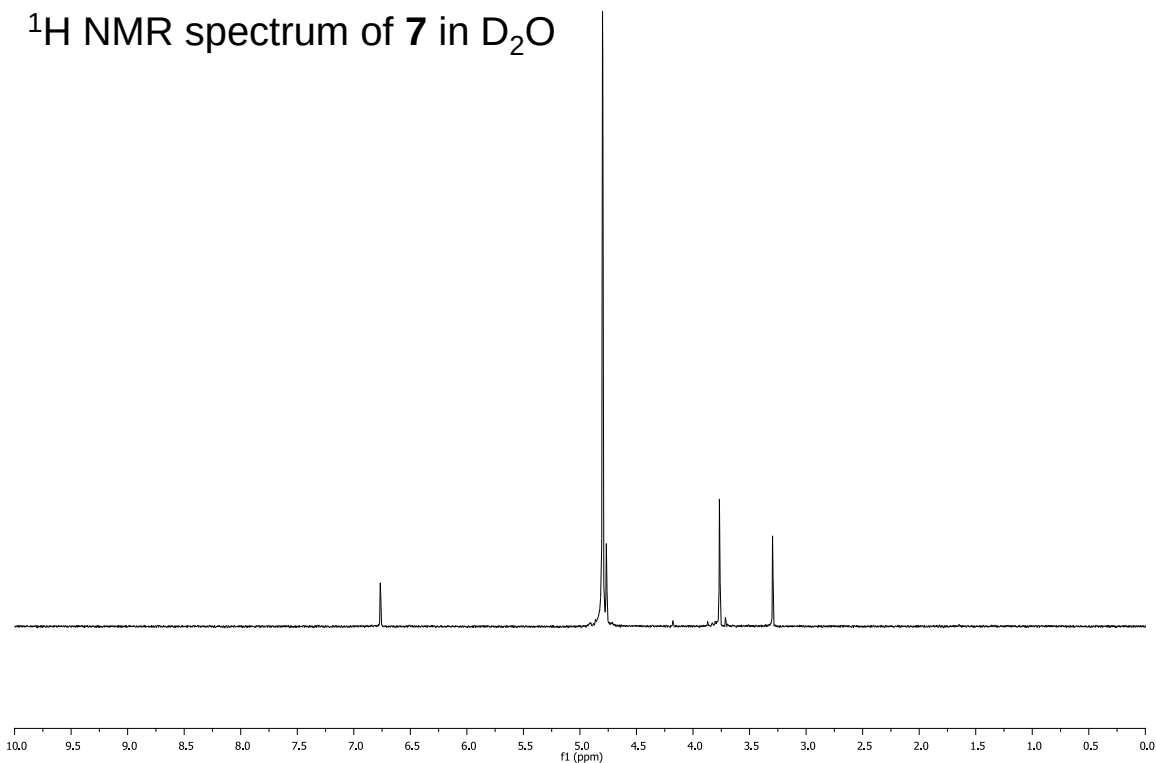




7

Dimethyl 2,2'-(4,5-diamino-1,2-phenylene)bis(oxy)diacetate • xHCl, 7. Under inert atmosphere, **4** (75.6 mg, 0.230 mmol) was dissolved in 20 mL dry methanol. The resulting solution was cooled to 0 °C, and thionyl chloride (0.034 mL, 0.459 mmol) was added dropwise. The reaction was allowed to reflux for 1 h and was then concentrated *in vacuo* to yield **7** as a purple solid (76.2 mg, 0.213 mmol, 93%). mp: 105 °C; ^1H NMR (300 MHz, D_2O) δ 6.77 (s, 2H), 4.77 (s, partially under solvent), 3.77 (s, 6H), 3.30 (s, 4H); ^{13}C NMR (101 MHz, D_2O) δ 171.59, 144.30, 123.17, 108.88, 66.66, 52.81; HRMS-ESI (m/z): $[\text{M}+\text{H}]^+$ calculated for $\text{C}_{12}\text{H}_{17}\text{N}_2\text{O}_6$ 285.10866; found, 285.10983.

^1H NMR spectrum of **7** in D_2O



(R)-3-(1,2-dihydroxyethyl)-2-methylquinoxaline-5-carboxylic acid and (R)-2-(1,2-dihydroxyethyl)-3-methylquinoxaline-5-carboxylic acid (DPD-BAQ), reaction with DPD followed by ^1H NMR. The known compound, **DPD**, was synthesized according to a previously reported procedure.¹⁹⁵ Briefly, (S)-4,5-cyclohexylidenedioxy-2,3-pentadione, **Pro-DPD**, (1.1 mg, 4.7 mmol) was fully dissolved in D_2O (1.1 mL), and D_2SO_4 (1.1 μL) was then added to the solution. Deprotection was then allowed to proceed for 3 h. Tag **2** (1 molar equiv, 1.2 mg, 4.7 mmol) was then added to the **DPD** solution, and the reaction was monitored by ^1H NMR for 1 h. At this time, a second molar equiv of **2** (1.2 mg, 4.7 mmol) was added to the reaction mixture, and monitoring by ^1H NMR was continued for another hour. After 2 h, a third molar equiv of **2** (1.2 mg, 4.7 mmol) was added to the reaction mixture, and monitoring by ^1H NMR was continued for another 0.5 h. After a total time of 2.5 h, conversion of **DPD** to **DPD-BAQ** was not complete, and the experiment was abandoned due to the sluggishness of the reaction. ^1H NMR (300 MHz, D_2O) characterized as a mixture of regioisomeric products. See Figure 7.4 for chemical shift assignments.

(R)-ethyl 3-(1,2-dihydroxyethyl)-2-methylquinoxaline-5-carboxylate and (R)-ethyl 2-(1,2-dihydroxyethyl)-3-methylquinoxaline-5-carboxylate (DPD-EBAQ), reaction with DPD followed by ^1H NMR. Known compound, **DPD**, was synthesized according to a previously reported procedure.¹⁹⁵ Briefly, **Pro-DPD** (1 mg, 4.7 mmol) was fully dissolved in D_2O (1 mL), and D_2SO_4 (1 μL) was then added to the solution. Deprotection was then allowed to proceed for 3 h. Tag **3** (1 molar equiv, 1.2 mg, 4.7 mmol) was then added to the **DPD** solution, and the reaction was monitored by ^1H NMR for 1 h. At this time, a second molar equiv of **3** (1.2 mg, 4.7 mmol) was added to the reaction mixture, and monitoring by ^1H NMR was continued for another 1 h. After a total time of 2 h, **DPD** had completely converted to **DPD-EBAQ** as judged by ^1H NMR. ^1H NMR (300 MHz, D_2O) characterized as a mixture of regioisomeric products. See Figure 7.4 for chemical shift assignments.

(R)-dimethyl 2,2'-(2-(1,2-dihydroxyethyl)-3-methylquinoxaline-6,7-diyl)bis(oxy)diacetate (DPD-M1CQ), Reaction with DPD Followed by ^1H NMR. The known compound, **DPD**, was synthesized according to a previously reported

procedure.¹⁹⁵ Briefly, **Pro-DPD** (1.1 mg, 4.7 mmol) was dissolved fully in D₂O (1.1 mL), and D₂SO₄ (1.1 μ L) was then added to the solution. Deprotection was then allowed to proceed for 3 h. Tag **7** (1 molar equiv, 1.9 mg, 4.7 mmol) was then added to the **DPD** solution, and the reaction was monitored by ¹H NMR for 0.5 h. At this time, a second molar equiv of **7** (1.9 mg, 4.7 mmol) was added to the reaction mixture, and monitoring by ¹H NMR was continued for another 0.5 h. After a total time of 1 h, **DPD** had completely converted to **DPD-M1CQ** as judged by ¹H NMR. ¹H NMR (300 MHz, D₂O) δ 7.58 (s, 1H), 7.36 (s, 1H), 5.28 (t, J = 5.5 Hz, 1H), 5.05 (d, J = 6.0 Hz, 4H), 3.95 – 3.71 (m, 2H, peak partially overlapping with the methyl ester protons from both **7** and **DPD-M1CQ**), 3.79 (s, 3H, peak overlapping with protons on **DPD-M1CQ**), 2.85 (s, 3H).

General Chromatographic Details. High performance liquid chromatography (HPLC) was performed utilizing a quaternary pump to generate a gradient for the elution of compounds from the stationary phase. For all samples, 10 μ L was injected onto the column via an autosampler cooled to 4 °C. A flow rate of 150 μ L/min was used, and the eluent was introduced directly into the MS for ion detection. The mobile phases were HPLC grade water (solvent A) and HPLC grade acetonitrile (solvent B), and these were used to construct the following 18 min gradient elution profile: t 0 min, 15% solvent A, 85% solvent B; t 2 min, 15% solvent A, 85% solvent B; t 4 min, 95% solvent A, 5% solvent B; t 14, 95% solvent A, 5% solvent B; t 16, 15% solvent A, 85% solvent B; t 18, 15% solvent A, 85% solvent B. The stationary phase used for these studies consisted of aminopropyl functionalized particles (5 μ m pore size, 100 Å particle size) packed into a 250 $\times$ 2 mm column (Phenomenex Luna NH2). All separations were performed with a column temperature of 10 °C.

General Mass Spectrometric Detection Parameters. All MS analyses were performed on a TSQ Quantum Discovery Max triple quadrupole MS (Thermo Electron Corporation). Samples were introduced into the electrospray ionization (ESI) chamber through a 0.1 mm internal diameter fused silica capillary after delivery by HPLC as described above, or by direct infusion via a syringe pump at a flow rate of 20 μ L/min. The spray voltage for the ESI source was set to 4500 V, and detection occurred in positive ion mode. Nitrogen was used as the sheath gas (10 psi), and the inlet capillary temperature was 300 °C. The argon used as the collision gas was set at 1.5 mTorr. The scan time for each SRM

was 0.05 s with a scan width of 1 m/z . All peaks from the mass chromatograms were integrated manually using the Xcalibur MS software package (Thermo Electron Corporation).

Preparation of Synthetic DPD (or (1-¹³C)DPD) for Biological Studies. This molecule was synthesized as described previously, and only general handling protocols are described. here. Fresh DPD was prepared daily from a stock solution of (S)-4,5-cyclohexylidenedioxy-2,3-pentadione (Pro-DPD) in water (1 mg/mL, 4.7 mM). This solution was routinely stored at 80 °C for a period of up to 3 weeks, and no change in the data were observed. When needed, 100 μ L aliquots of the Pro-DPD solution were prepared by addition of 1 μ L 10% H₂SO₄. Deprotection was allowed to proceed for 3 h at ambient temperature, and the resulting DPD solution (4.7 mM) was used without modification for further experimentation.

Bacterial Strains and Growth Conditions. The *E. coli* wild type strain BW25113 and *luxS*⁻ strain JW2662-1 were established as part of the Keio Collection¹⁹⁶ and were purchased from the Coli Genetic Stock Center at Yale University. For all experiments, *E. coli* cultures were grown in Luria Bertani medium (LB)¹⁸⁹ at 37 °C with aeration. The *V. harveyi* wild type strain BB120¹⁵⁴ and *luxS*⁻ strain MM30¹⁵⁵ were a gift from B.L. Bassler (Princeton University and the Howard Hughes Medical Institute). Overnight cultures of *V. harveyi* BB120 were grown in Luria Marine medium (LM)¹⁵³ at 37 °C with aeration. Overnight cultures of *V. harveyi* MM30 were cultured in LM supplemented with 100 mg/L kanamycin. For experiments in which the [DPD] was to be determined, both *V. harveyi* strains were cultured in LM at 30 °C with aeration.

General Procedure for the Determination of the [DPD]. For the detection of DPD in samples of *E. coli* or *V. harveyi*, 300 μ L aliquots were taken at regular intervals from duplicate cell cultures of both the wild type and *luxS*⁻ strain grown as described above. Single 300 μ L aliquots for each sample of saliva were generated as previously described. These aliquots were then added to a 1.5 mL microcentrifuge tube containing a solution of the internal standard, (1-¹³C)DPD (341 μ M in 10 μ L H₂O for bacterial samples, 34.1 μ M in 10 μ L H₂O for saliva samples). The resulting solution was thoroughly mixed and then centrifuged (13,200 rpm, 16,100 rcf, 1 min for bacterial

samples, 5 min in for saliva samples) to remove cells and other particulates. A portion of the resulting supernatant (200 μ L) was then transferred to a 300 μ L screwcap autosampler vial containing a solution of tag 7 (14 mM in 20 μ L H₂O), and the two liquids were thoroughly mixed. After 1 h incubation at ambient temperature, the samples were quickly frozen and then stored at -80 °C until no more than 1.5 h prior to MS analysis. Duplicate injections of each saliva sample were performed. Two SRMs, 381-202 and 382-203, were used to determine the relative signal arising from detection of **DPD-M1CQ** to that of **(13C)DPD-M1CQ**. After manual peak integration, the data were corrected for isotopic impurities and the following equation was used to calculate the [DPD]:

$$\frac{\text{Signal DPD-M1CQ}}{\text{Signal } (1^{13}\text{C})\text{DPD-M1CQ}} \times [(1^{13}\text{C})\text{DPD in solution A}] \times \frac{\text{volume solution A}}{\text{volume culture sampled}} = [\text{DPD}]$$

Comparison of the Ionization Efficiencies for the Quinoxalines Derived from Reaction of Diamine Tags 2-7 with Synthetic DPD. To generate each quinoxaline for study, an ~25 μ L solution of the tagging reagent (14 mM in H₂O) was mixed in a 1:1 v/v ratio with a solution of DPD (4.7 mM in H₂O, pH = 1.8) to give a solution containing a 3:1 mixture of the diamine tag to DPD. After the reactions were allowed to proceed for 2 h at ambient temperature, each of the 6 resulting solutions were diluted in water to a final concentration of 10 μ M. Determination of the relative ionization efficiency for each was then accomplished via LC-MS/MS analysis utilizing the appropriate SRM detection parameters. See Figure 7.3 for results.

Determination of the Approximate Linear Range and Limit of Detection for DPD-M1CQ. To determine the ability to detect **DPD-M1CQ** via LC-MS/MS, an ~200 μ L solution of the tagging reagent (14 mM in H₂O) was mixed in a 1:1 ratio with a solution of DPD (4.7 mM in H₂O, pH = 1.8) to give a solution containing a 3:1 mixture of the diamine tag to DPD. After reaction was allowed to proceed for 30 min at ambient temperature, a 1:100 dilution of the sample was performed to give a 23.5 μ M solution of **DPD-M1CQ** in H₂O. Further half log-dilutions of the molecule in water gave samples with a series of concentration ranging from 74 pM to 7.4 mM. For this experiment, samples were stored at -80 °C until no more than 5 h before LC-MS/MS analysis. The total ion counts measured via each of the appropriate SRMs was determined by integrating the peak for

DPD-M1CQ in each mass chromatogram. The results of these data are summarized in Figure 7.5.

Validation of Selectivity for Tag 7 in Complex Media. Three aliquots of sterile LB (200 μ L) were mixed with a solution of tag 7 (14 mM in 20 μ L H₂O). After thorough mixing, the solutions were allowed to incubate for 1 h at ambient temperature to mimic the conditions used for the reaction of DPD with 7. To determine whether reaction of 7 with components in the media had occurred, the samples were analyzed via LC-MS/MS, and control samples containing only LB were also analyzed. During these experiments, the SRMs 381-201, 381-202, and 381-231 that were projected to be optimal for the detection of DPD were monitored. No signal was observed above background in any samples containing only LB in the SRMs used to detect either **DPD-M1CQ** or (¹³C)**DPD-M1CQ**.

Comparison of Internal Standards (¹³C)DPD-M1CQ and (1-¹³C)DPD. To determine the relative efficacy of these internal standards, the [DPD] from a culture of *V. harveyi* BB120 grown for 9 h was measured in duplicate relative to either (¹³C)**DPD-M1CQ** or (1-¹³C)**DPD**. The samples were prepared as described in the General Procedure for the Determination of the [DPD] entry in the methods section, except that a solution of (¹³C)**DPD-M1CQ** (341 μ M in H₂O) was substituted as the internal standard for two of the samples. The initial (¹³C)**DPD-M1CQ** solution (2.35 mM in H₂O) was produced by mixing a solution of 7 (14 mM in H₂O) with (1-¹³C)**DPD** (4.7 mM in H₂O) in a 1:1 v/v ratio and allowing the reaction to proceed for 1 h at ambient temperature.

Determination of the [DPD] in *E. coli* Wild Type Strain BW25113 and *luxS*⁻ Strain JW2662-1. A culture of both strains was grown overnight, and then used to make two 2% (v/v) inocula of each in fresh LB (25 mL) in 125 mL glass Erlenmeyer flasks as described in the *Bacterial Strains and Growth Conditions* entry in the methods section of this document. At the time intervals indicated in the table below, the cell density was determined by measuring the OD₆₀₀ for 1 mL aliquots of each culture, and separate 300 μ L aliquots were used for measurement of the [DPD]. Samples were prepared and analyzed as described in the *General Procedure for the Determination of the [DPD]* section. Only one of the cultures for the *luxS*⁻ strain was fully analyzed as no DPD was

detected, although several of the samples from random times in the second culture were used to confirm the lack of signal.

Determination of the [DPD] in *V. harveyi* Wild Type Strain BB120 and *luxS*⁻ Strain MM30. A culture of both strains was grown overnight, and then used to make two 2% (v/v) inocula of each in fresh LM (25 mL) in 125 mL glass Erlenmeyer flasks as described in the *Bacterial Strains and Growth Conditions* entry in the methods section of this document. At the time intervals indicated in the table below, the cell density was determined by measuring the OD₆₀₀ for 1 mL aliquots of each culture, and separate 300 µL aliquots were used for measurement of the [DPD]. Samples were prepared and analyzed as described in the *General Procedure for the Determination of the [DPD]* section. Only one of the cultures for the *luxS*⁻ strain was fully analyzed as no **DPD** was detected, although several of the samples from random times in the second culture were used to confirm the lack of signal.

Collection of Saliva Samples. Volunteers were each asked to deposit ~1 mL of mucus free saliva into a specimen cup. The sample was then agitated to remove bubbles, and the saliva was then used for further experimentation as described in the *General Procedure for the Determination of the [DPD]* section.

CHAPTER VIII

A QUANTITATIVE DEFINITION OF QUORUM SENSING AND ITS EXTENSION TO A VARIETY OF SPECIES

Some of the data presented below has been adapted from the following published journal article:

Jessica R. Gooding, Amanda L. May, Kathryn R. Hilliard, and Shawn R. Campagna. Establishing a Quantitative Definition of Quorum Sensing Provides Insight into the Information Content of the Autoinducer Signals in *Vibrio harveyi* and *Escherichia coli*. *Biochemistry* **2010**, 49, 5621-5623. JRG performed the [DPD] assays, glucose assays and the DPD flux assays. ALM synthesized deuterated HAI-1, setup the HPLC detection method and performed the assays. KRH aided some of the preliminary *E. coli* wild type [DPD] assays. SRC contributed to the experimental design and interpretation of the data. DOI: [10.1021/bi1001163](https://doi.org/10.1021/bi1001163). Copyright © 2010 American Chemical Society

8.1 Background

Many species of bacteria produce more than one quorum sensing signal, and since the discovery of a dual system in *Vibrio harveyi*, researchers have been trying to explain why.¹⁵³ The two quorum sensing signals AI-2 and HAI-1 are detected by a converging set of signaling cascades with shared regulatory components,¹⁵³ and thus it is unclear how the bacterium is able to distinguish between the two. Currently proposed mechanisms for signal differentiation include a difference in timing of the biosynthesis of each molecule¹⁹⁷ and/or a difference in signal receptor expression ratios.¹⁹⁸

Due, in part, to a poor understanding of the signal integration, the information content of these molecules is also uncertain. One explanation offered is that HAI-1 may report “self” while AI-2 reports “others.”¹⁵⁴ Alternatively, the signals may be better interpreted as indicators of community development stage.¹⁹⁹ We hypothesize that the information content of a signal will be determined by the regulation of biosynthesis or secretion of the signal. If a signal were not regulated in this manner, the concentration of the signal would be subject to inference from the changing environment of the organism and the information carried would be lost in environmental noise.

Quorum sensing signals on a phenotypic level regulate cell density dependent phenotypes. On a molecular level, a population may achieve this one of two ways. First, using a “switch” mechanism, the population could produce little quorum sensing signal at low cell densities and then abruptly switch on high-density phenotypes after a surge in biosynthesis. On the other hand, the population could rely on a “graded” mechanism where the population gradually expresses quorum sensing signal overtime in keeping with cell density. We propose that a gradual build in autoinducer concentration in proportion to cell density is the simplest means to carry cell density information and requires no deconvolution on the part of the bacteria. This system would also be less vulnerable to metabolic interference and other noise. This hypothesis can be described by the equation:

$$\frac{d(\text{extracellular signal concentration})}{d(\text{cell density})} = \text{constant}$$

We propose the relationship above as a quantitative definition of cell-density dependent quorum sensing, and provide evidence of its validity in the following set of experiments.

8.2 Validation of the Definition Using Two Model Systems

To test this definition, two model systems *E. coli* and *V. harveyi* were chosen because they are well characterized and are archetypes of the two extremes of the known AI-2 signaling systems (see Chapter 6). Because the nutritional environment can be expected to interfere with signaling and particularly because of the tie between DPD biosynthesis and metabolism, a series of glucose concentrations was chosen to probe the effect of nutrient availability on DPD production. Cultures of each species were inoculated in fresh media, either LB or LM, supplemented with 0, 0.08, 0.14, or 0.2 w/v % glucose and the supernatants were tested for [DPD] over time into stationary phase via methods described in Chapter 7. Glucose is an atypical source of carbon for *V. harveyi*, so glycerol experiments were also run as a control. The results of the *V. harveyi* BB120 glucose, *V. harveyi* BB120 glycerol, and *E. coli* BW25113 glucose experiments can be found in Figures 8.1, 8.2, and 8.3 respectively.

V. harveyi utilizes two other quorum sensing signals, a homoserine lactone HAI-1 and an α -hydroxyundecenone similar to the CAI-1 of *V. cholerae*.²⁰⁰ We undertook testing our definition with a second major set of quorum sensing signals using HAI-1. A

chromatography method was developed to measure AHLs, and deuterated HAI-1 internal standard was synthesized to support these studies (ALM and MEE). The ketone contributes much less to the expression of the high-density phenotype in this species, so with this in mind as well as the efforts needed to develop a new method, we decided not to pursue measurement of the α -hydroxyundecenone. For these experiments, overnight cultures of *V. harveyi* were similarly inoculated into fresh LM supplemented with 0, 0.08, 0.14, or 0.2 w/v % glucose and [HAI-1] was measured over time (Figure 8.4).

Concentrations of DPD reach 21 μ M in *V. harveyi* with no added carbon sources, and 24 μ M for 0.08%, whereas the concentration only reaches 14 μ M and 12 μ M for the higher amounts of added glucose. Likewise, HAI-1 concentrations rose to 29 and 24 μ M for 0.00 and 0.08% added glucose while higher concentrations only made 7 and 6 μ M for 0.14% and 0.20% added respectively. One possible explanation for this difference is the acidification of the media followed by cell death. However metabolic activity was evident in the glucose assay at all concentrations (with the exception of 0.20% glucose after 5.75 h in two of the four replicates), and a viability assay at 6.5 h showed 2.5×10^8 CFU/ml for the 0.14% added glucose culture, although this is almost an order of magnitude less than the no added glucose control. Optical density also demonstrated retarded growth in the high glucose cultures, and DPD production rates dropped steeply between 4.25 and 5 h. HAI-1 production dropped soon after between 5 and 5.75 h.

V. harveyi cultures supplemented with glycerol reached similar DPD concentrations when compared to each other, 20 μ M, 21 μ M and 21 μ M for 0.00, 0.07 and 0.17% w/v glycerol respectively, as well as when compared to glucose cultures. Unlike the glucose experiments, there was no observed difference in cell density between cultures.

E. coli strain BW25113 reached peak DPD concentrations of 19, 60, 86, and 130 μ M for 0.00, 0.08, 0.14 and 0.20 w/v% added glucose cultures respectively. Cell densities were consistent between samples. Supplemented glucose was consumed prior to 3.5 h for all cultures, and DPD production rates overlay glucose consumption neatly.

With these data in hand, we plotted three dimensional landscapes with the nutrient and time dimensions, against the autoinducer:cell density ratio of our proposed definition. Immediately obvious is the characteristic flat plane of DPD/OD₆₀₀ in *V. harveyi*, indicating that the bacteria regulate production of DPD in a way that holds DPD/cell density constant even under stress. In this species, our quantitative definition of quorum sensing holds true in every dimension for both carbon sources. This finding

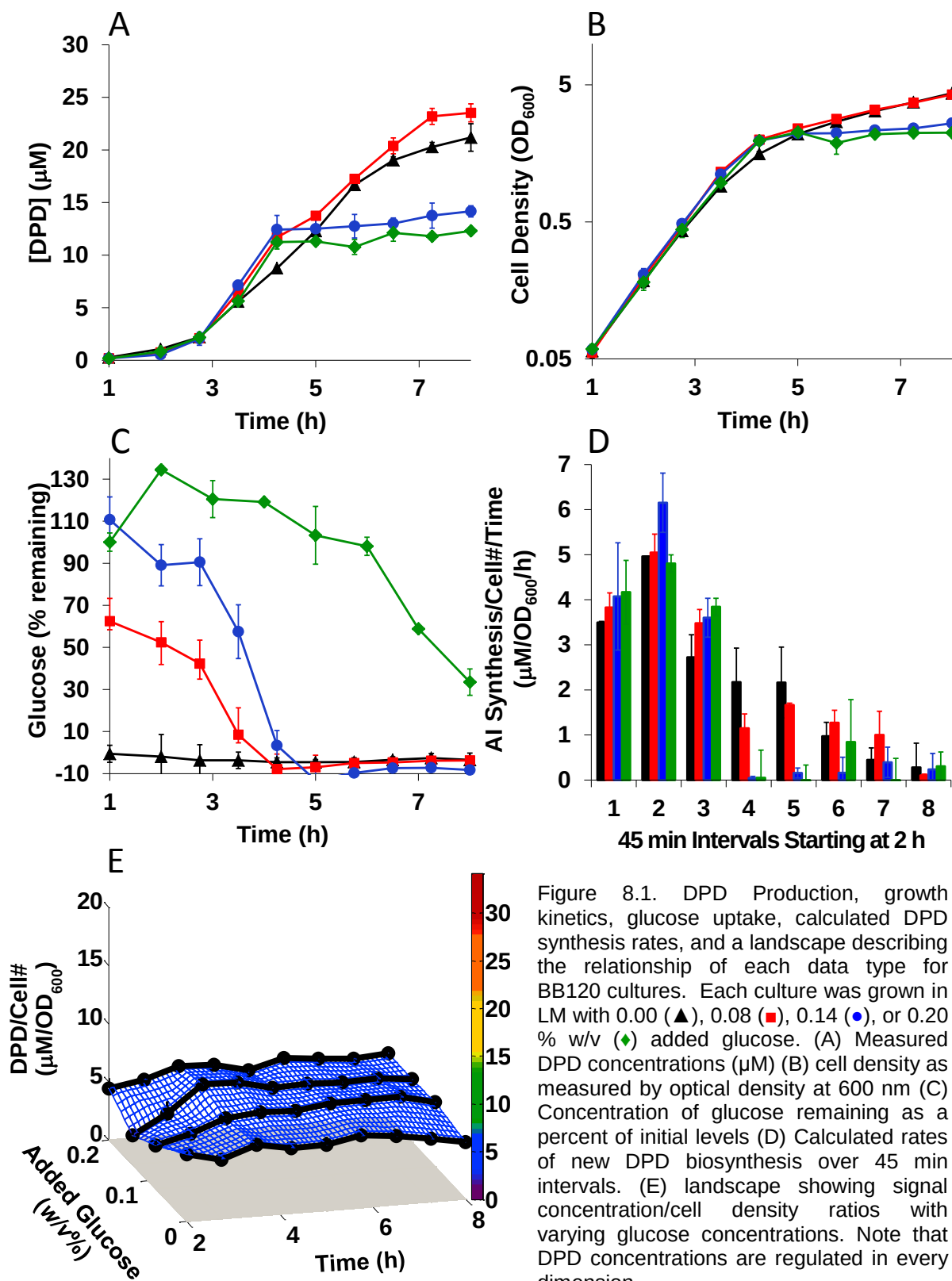


Figure 8.1. DPD Production, growth kinetics, glucose uptake, calculated DPD synthesis rates, and a landscape describing the relationship of each data type for BB120 cultures. Each culture was grown in LM with 0.00 ($\blacktriangle$), 0.08 ($\color{red}\blacksquare$), 0.14 ($\bullet$), or 0.20 % w/v ($\blacklozenge$) added glucose. (A) Measured DPD concentrations (μM) (B) cell density as measured by optical density at 600 nm (C) Concentration of glucose remaining as a percent of initial levels (D) Calculated rates of new DPD biosynthesis over 45 min intervals. (E) landscape showing signal concentration/cell density ratios with varying glucose concentrations. Note that DPD concentrations are regulated in every dimension.

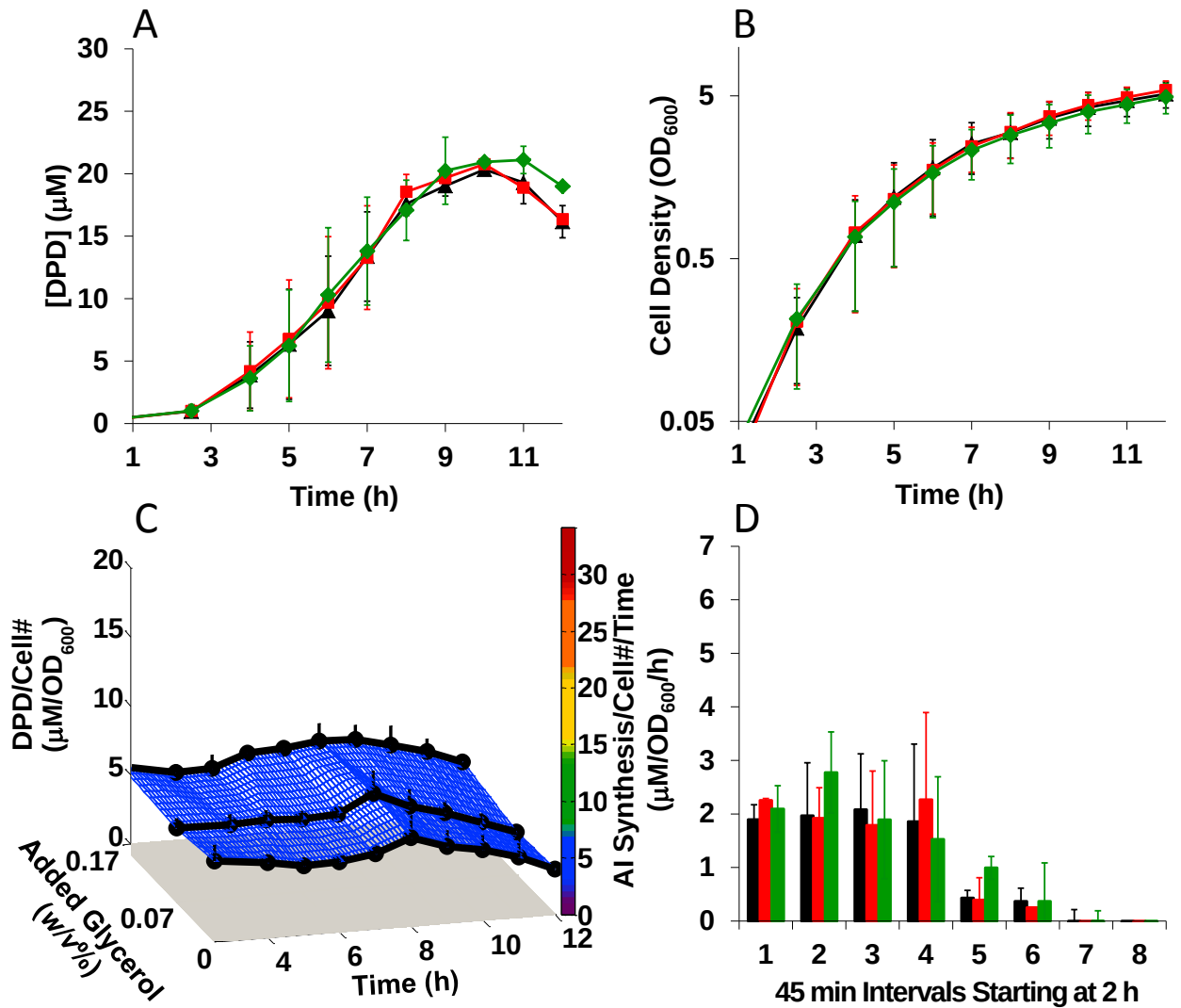


Figure 8.2. DPD Production, growth kinetics, calculated DPD synthesis rates, and a landscape describing the relationship of each data type for BB120 cultures. Each culture was grown in LM with 0.00 ($\blacktriangle$), 0.07 ($\color{red}\blacksquare$), or 0.17 % w/v ($\color{green}\blacklozenge$) added glycerol. (A) Measured DPD concentrations (μM) (B) cell density as measured by optical density at 600 nm (C) landscape showing signal concentration/cell density ratios with varying glycerol concentrations (D) Calculated rates of new DPD biosynthesis over 45 min intervals. Note that DPD concentrations are regulated in every dimension.

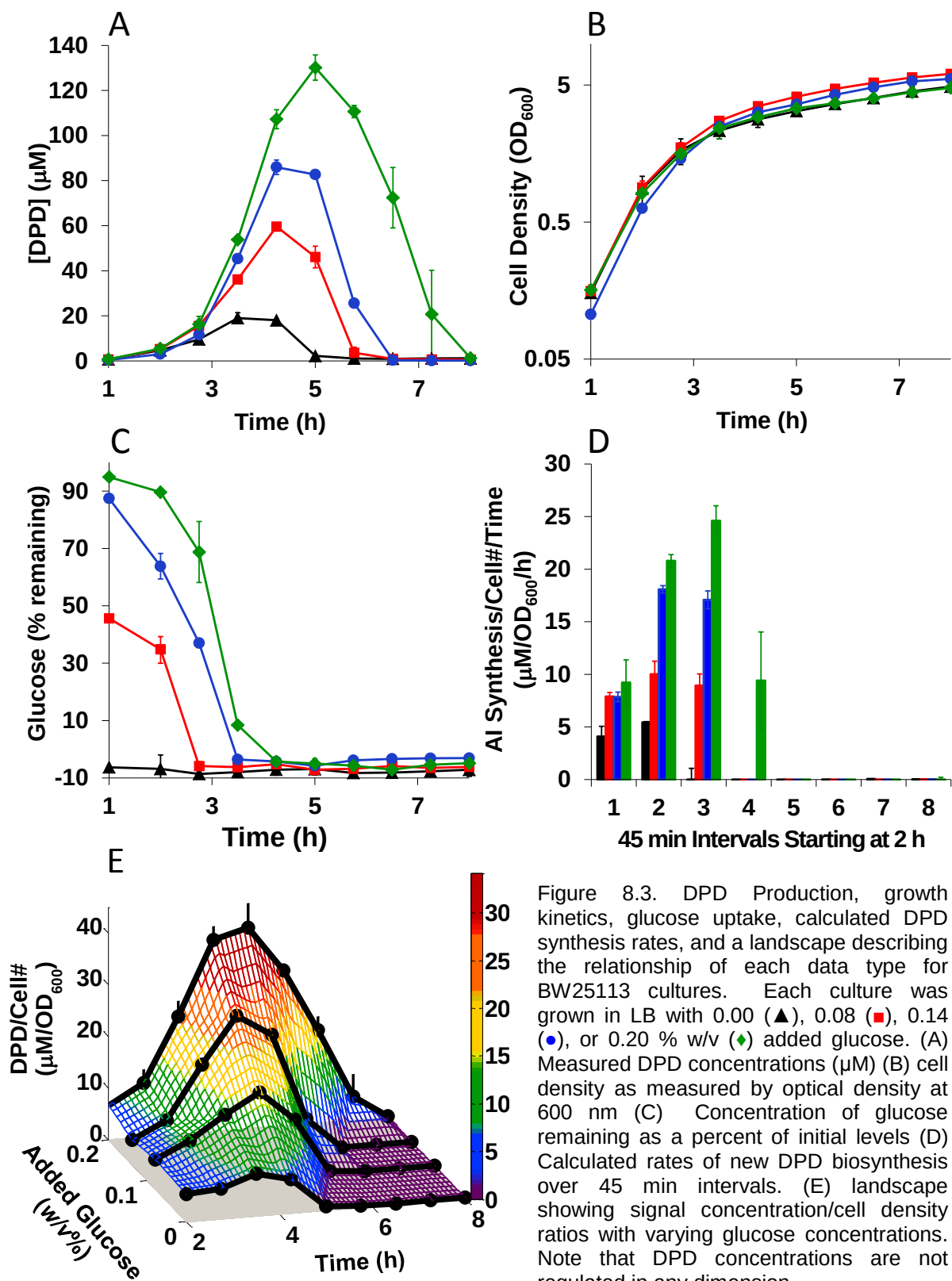


Figure 8.3. DPD Production, growth kinetics, glucose uptake, calculated DPD synthesis rates, and a landscape describing the relationship of each data type for BW25113 cultures. Each culture was grown in LB with 0.00 ($\blacktriangle$), 0.08 ($\color{red}\blacksquare$), 0.14 ($\bullet$), or 0.20 % w/v ($\blacklozenge$) added glucose. (A) Measured DPD concentrations (μM) (B) cell density as measured by optical density at 600 nm (C) Concentration of glucose remaining as a percent of initial levels (D) Calculated rates of new DPD biosynthesis over 45 min intervals. (E) landscape showing signal concentration/cell density ratios with varying glucose concentrations. Note that DPD concentrations are not regulated in any dimension.

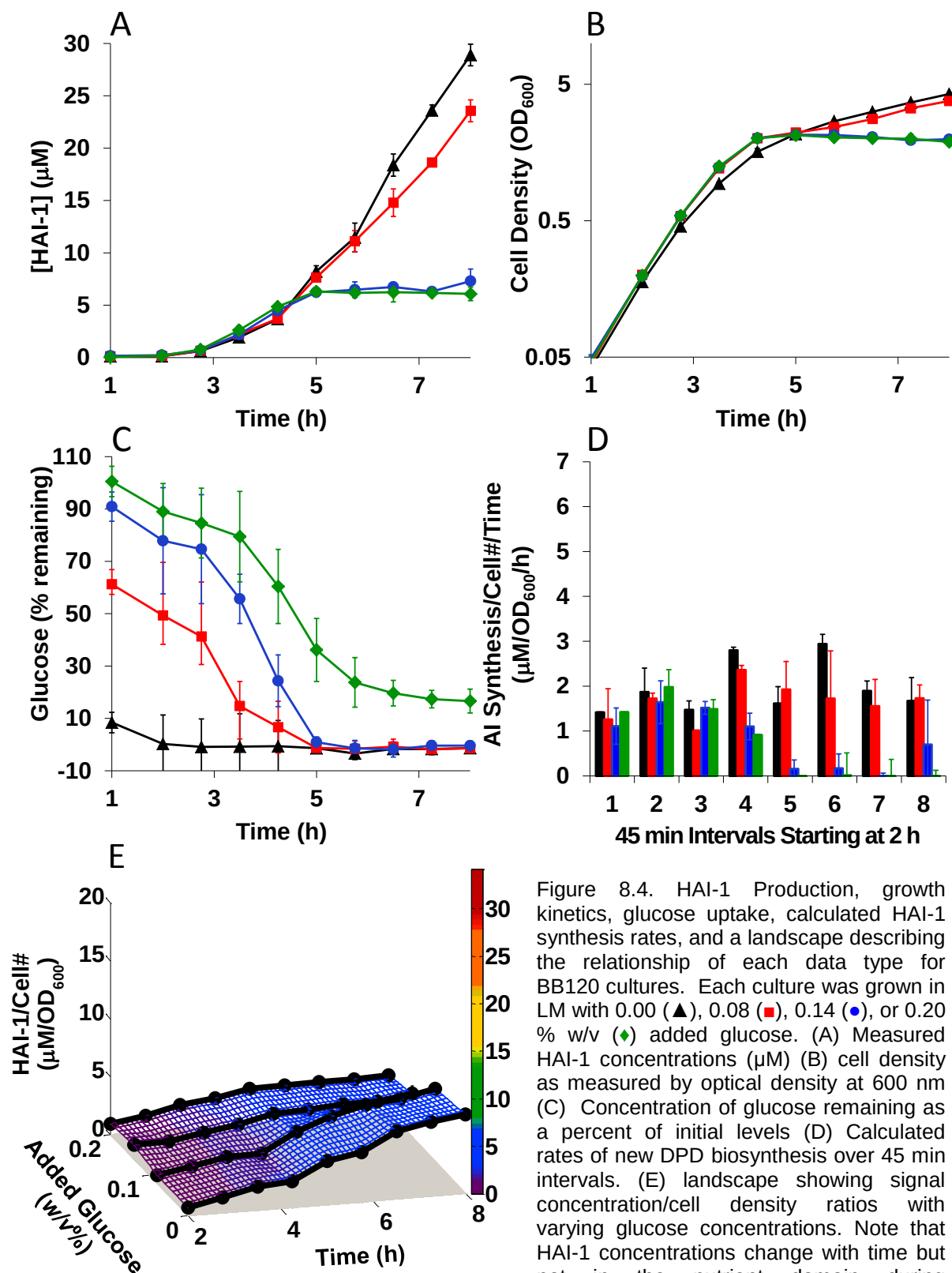


Figure 8.4. HAI-1 Production, growth kinetics, glucose uptake, calculated HAI-1 synthesis rates, and a landscape describing the relationship of each data type for BB120 cultures. Each culture was grown in LM with 0.00 ($\blacktriangle$), 0.08 ($\color{red}\blacksquare$), 0.14 ($\bullet$), or 0.20 % w/v ($\blacklozenge$) added glucose. (A) Measured HAI-1 concentrations (μM) (B) cell density as measured by optical density at 600 nm (C) Concentration of glucose remaining as a percent of initial levels (D) Calculated rates of new DPD biosynthesis over 45 min intervals. (E) landscape showing signal concentration/cell density ratios with varying glucose concentrations. Note that HAI-1 concentrations change with time but not in the nutrient domain during exponential phase.

validates our hypothesis since most of the data supporting AI-2 as signal was performed on *V. harveyi*.^{153,154} On the other hand, *E. coli* BW25113 does not regulate DPD production in any dimension, signifying that DPD and by extension AI-2 is not a quorum sensing signal in this species. Again this finding validates our definition and builds more evidence against the signaling role for AI-2 in *E. coli* that others have already called into question.¹⁶⁵

In contrast to the production of DPD, the ratio of HAI-1/OD₆₀₀ does climb over time in *V. harveyi*, but does not change considerably with glucose addition until 5 h, when the culture is under stress in stationary phase. We interpret this to mean that the information content of HAI-1 is actually metabolic in nature. This may be better illustrated by the rate of production data (Figure 8.4 panel D). The HAI-1 production rate is constant over time (~1.8 $\mu\text{M}/\text{OD}_{600}/\text{h}$) which is consistent with continued metabolic activity even into stationary phase, whereas DPD production rate slows upon entry into stationary phase (5 to 0.3 $\mu\text{M}/\text{OD}_{600}/\text{h}$) consistent with a correction for a decrease in cell division. Thus, we believe *V. harveyi* is able to use these signals to confer information about the cell density and metabolic health of the culture. Evidence in the literature suggests *V. harveyi* is able to determine a ratio of DPD to HAI-1 and this may be the mechanism for interpreting the different signals.¹⁹⁹

Beyond the evidence concerning information content, we learned that neither signal is produced in a switch-like manner, supporting earlier work.^{199,201} We also found that induction of HAI-1 production occurred about 45 min after DPD appeared, but both were measurable prior to densities where high density phenotypes are normally observed.²⁰²

Other than regulation of its own production and consumption,¹⁷³ AI-2 quorum sensing has really only been tied to pathogenicity in *E. coli*, and even this role is not uncontested.²⁰³ However, it seemed prudent to screen something other than a lab-adapted, non-pathogenic strain of *E. coli* in order to fully address the question. To this end, a series of wild type isolates from the ATCC biological resource center were also characterized: 8739 (an *E. coli* C), 25404 (K-12), 25922 (a smooth strain), and 700728 (a nontoxigenic O157:H7) (Figures 8.5-8.8 respectively). A mutant of BW25113 incapable of transporting DPD into the cell (*IsrB*⁻) was also screened as a means to observe DPD production decoupled from DPD consumption (Figure 8.9).

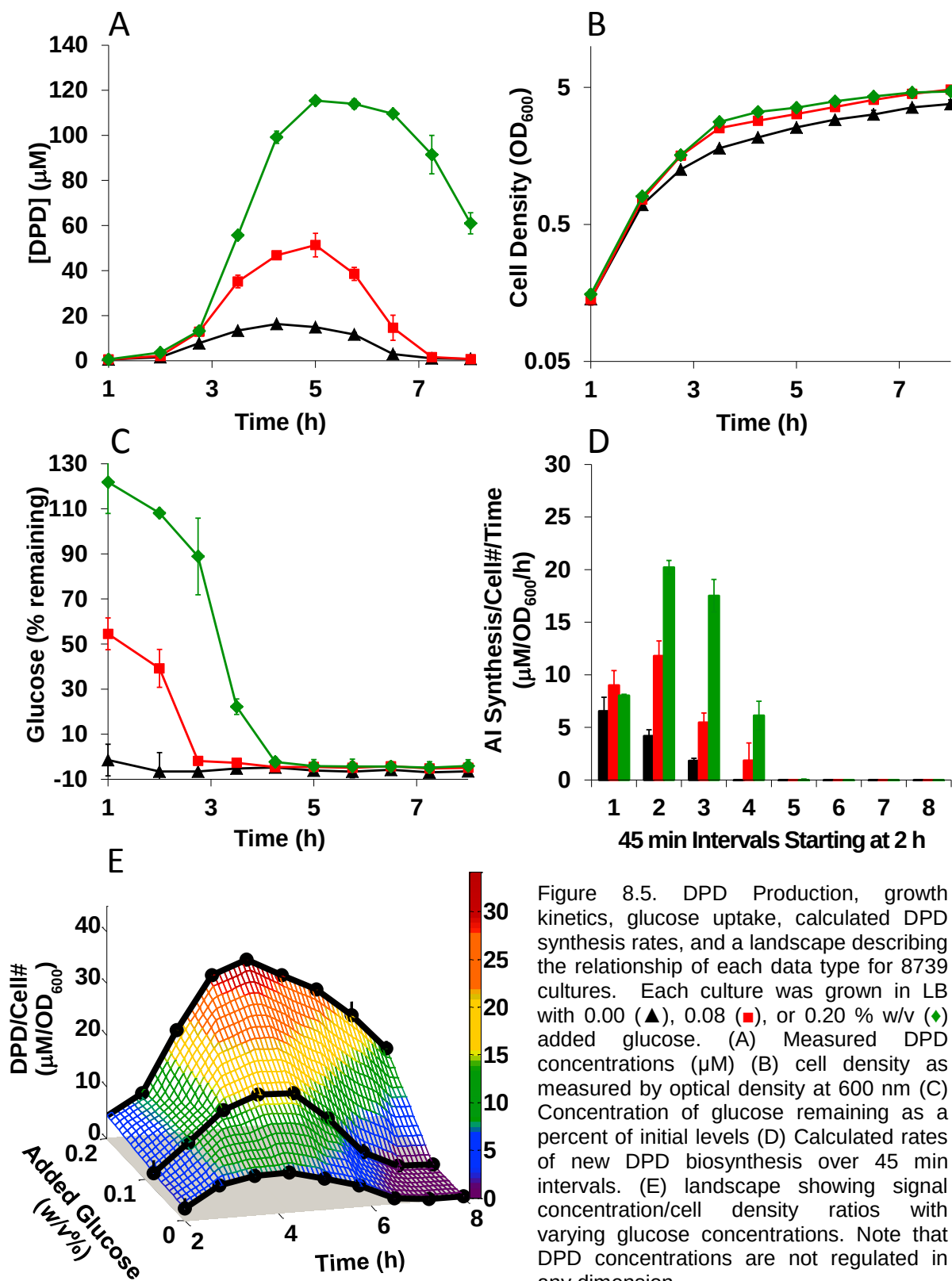
Each of these strains had characteristic differences when compared to BW25113. ATCC 8739 was problematic to handle because it produced some type of emulsifier that prevented sample extraction. This strain peaked at 16, 51, and 115 μM DPD for 0.00, 0.08, and 0.20 w/v% added glucose cultures respectively and glucose was absorbed at a comparable rate to BW25113. Interestingly, the peak in DPD production was less steep, and it took 8739 both longer to reach peak production, and longer to import a smaller amount of DPD (Figure 8.5). One could argue that DPD biosynthesis is reasonably well regulated in the no added glucose culture.

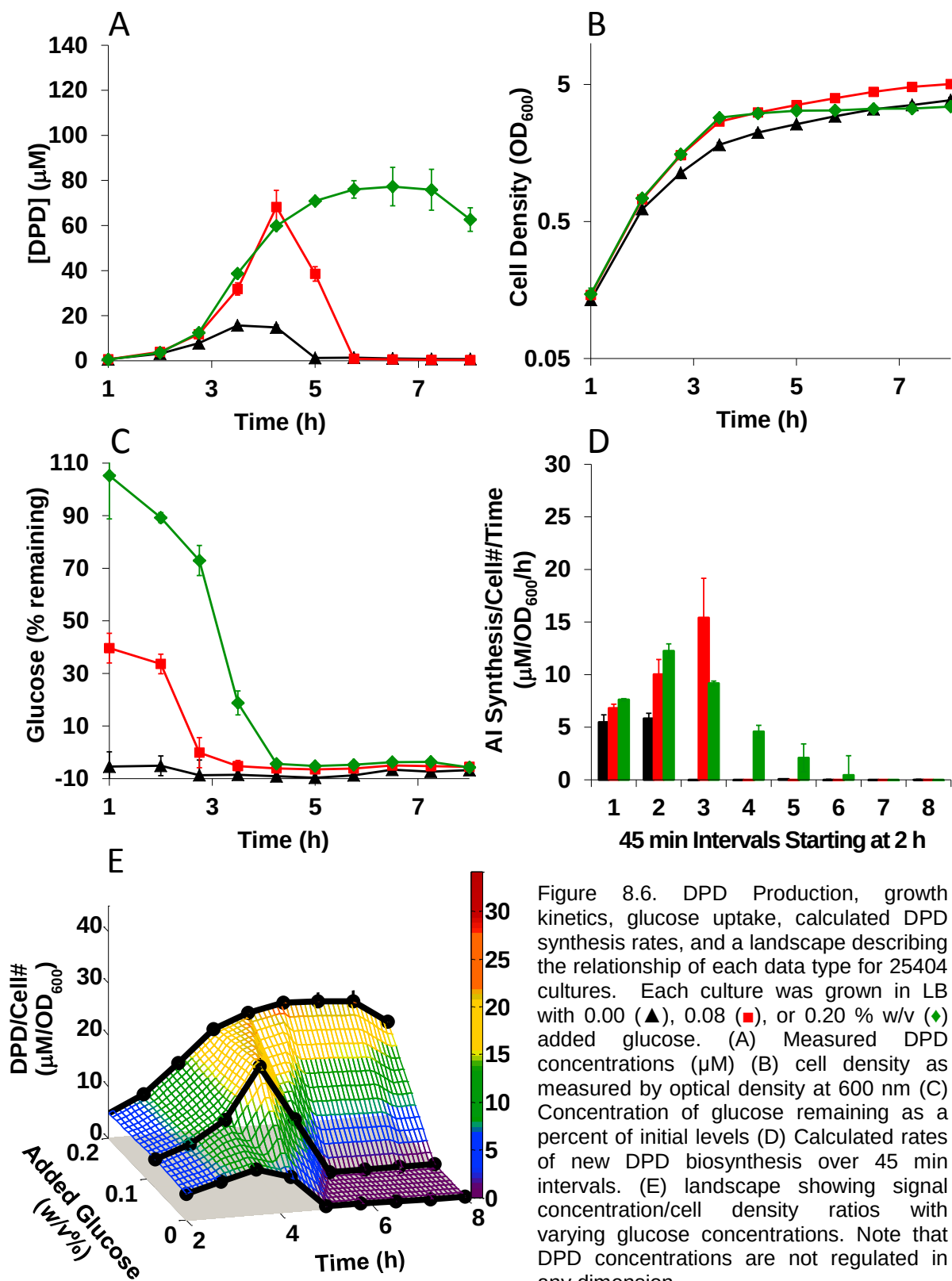
ATCC 25404 reached 16, 68 and 77 μM DPD for 0.00, 0.08, and 0.20 w/v% added glucose cultures respectively. The 0.08% glucose culture peaked sharply at 4.25 h like BW25113. However, instead of continuing to climb in DPD concentration the 0.20% glucose sample leveled off at about the same concentration, becoming the producer of the smallest DPD concentration at high added glucose (Figure 8.6). This strain was very slow to import DPD at high glucose concentrations showing no definitive signs of importation at 8 h.

ATCC 25922 also had some unique technical challenges. During the early exponential phase, the culture flocculates, making it challenging to get a good optical density reading and increasing error in the early part of the experiment. The strain has peak DPD concentrations of 21, 48 and 129 μM DPD for 0.00, 0.08, and 0.20 w/v% added glucose cultures respectively. The cultures exhibited so little DPD importation in 8 h that the experiment was extended to 15 h in order to ascertain whether it had a functional Lsr transport complex (Figure 8.7).

ATCC 700728 produced peak DPD concentrations at 16, 44, and 107 μM for 0.00, 0.08, and 0.20 w/v% added glucose cultures respectively. The rate of growth, glucose consumption and DPD production were typical when compared to the other strains. This strain, being genetically closest to the pathogenic species, did not display any new or unique activity in these assays (Figure 8.8).

The BW25113 mutant strain JW1509-1 (*lsrB*⁻) produced more DPD than the parent strain BW25113 at all glucose concentrations, reaching 42, 93, 147 μM for 0.00, 0.08, and 0.20 w/v% added glucose cultures respectively. The growth rate was similar to the other strains. While DPD production continued somewhat later in the growth cycle than the parent strain, one may note however the concentration did peak (Figure 8.9). DPD was removed from solution either by degradation or by an alternate importation





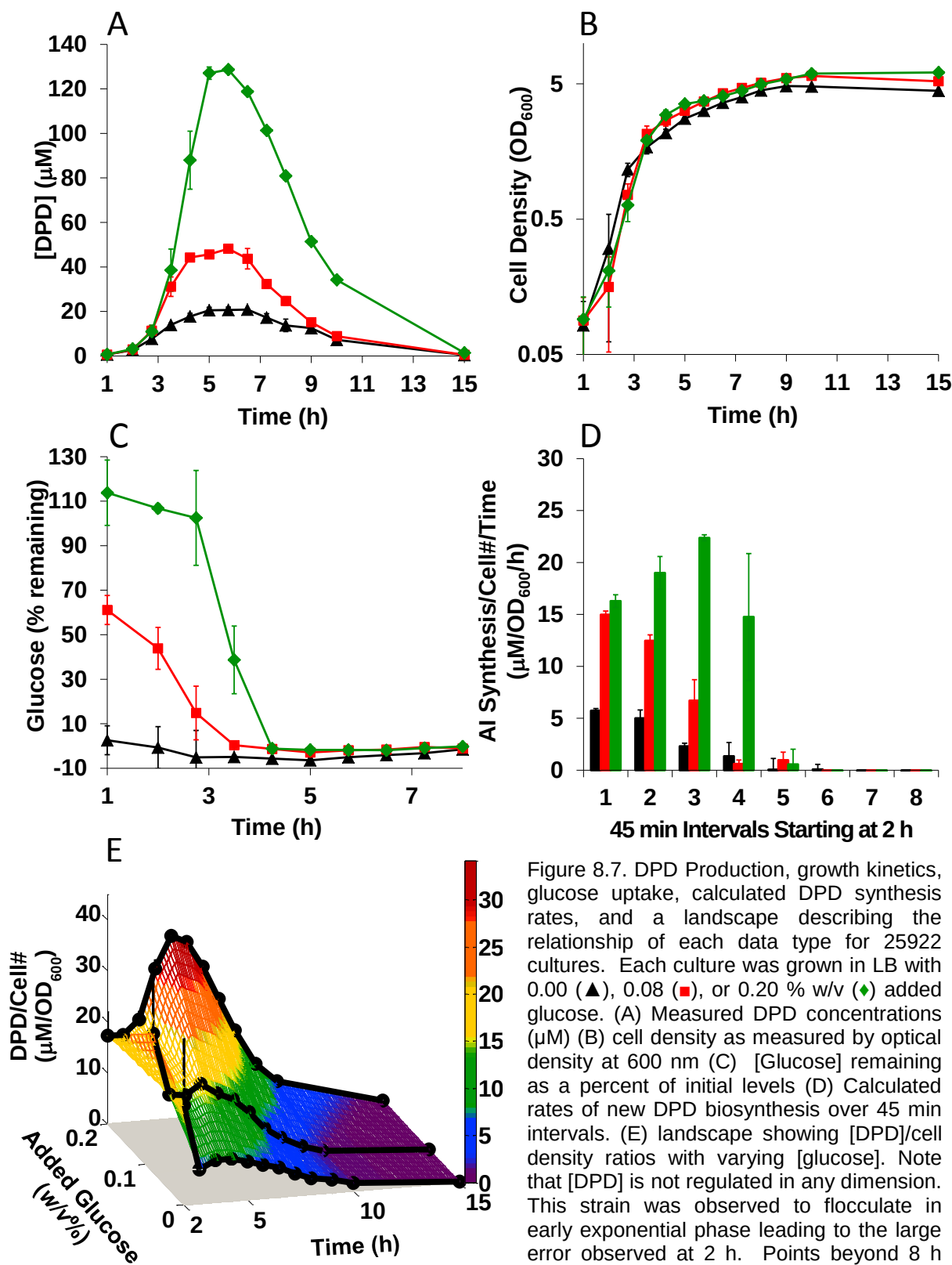


Figure 8.7. DPD Production, growth kinetics, glucose uptake, calculated DPD synthesis rates, and a landscape describing the relationship of each data type for 25922 cultures. Each culture was grown in LB with 0.00 ($\blacktriangle$), 0.08 ($\color{red}\blacksquare$), or 0.20 % w/v ($\color{green}\blacklozenge$) added glucose. (A) Measured DPD concentrations (μM) (B) cell density as measured by optical density at 600 nm (C) [Glucose] remaining as a percent of initial levels (D) Calculated rates of new DPD biosynthesis over 45 min intervals. (E) landscape showing [DPD]/cell density ratios with varying [glucose]. Note that [DPD] is not regulated in any dimension. This strain was observed to flocculate in early exponential phase leading to the large error observed at 2 h. Points beyond 8 h have not been repeated.

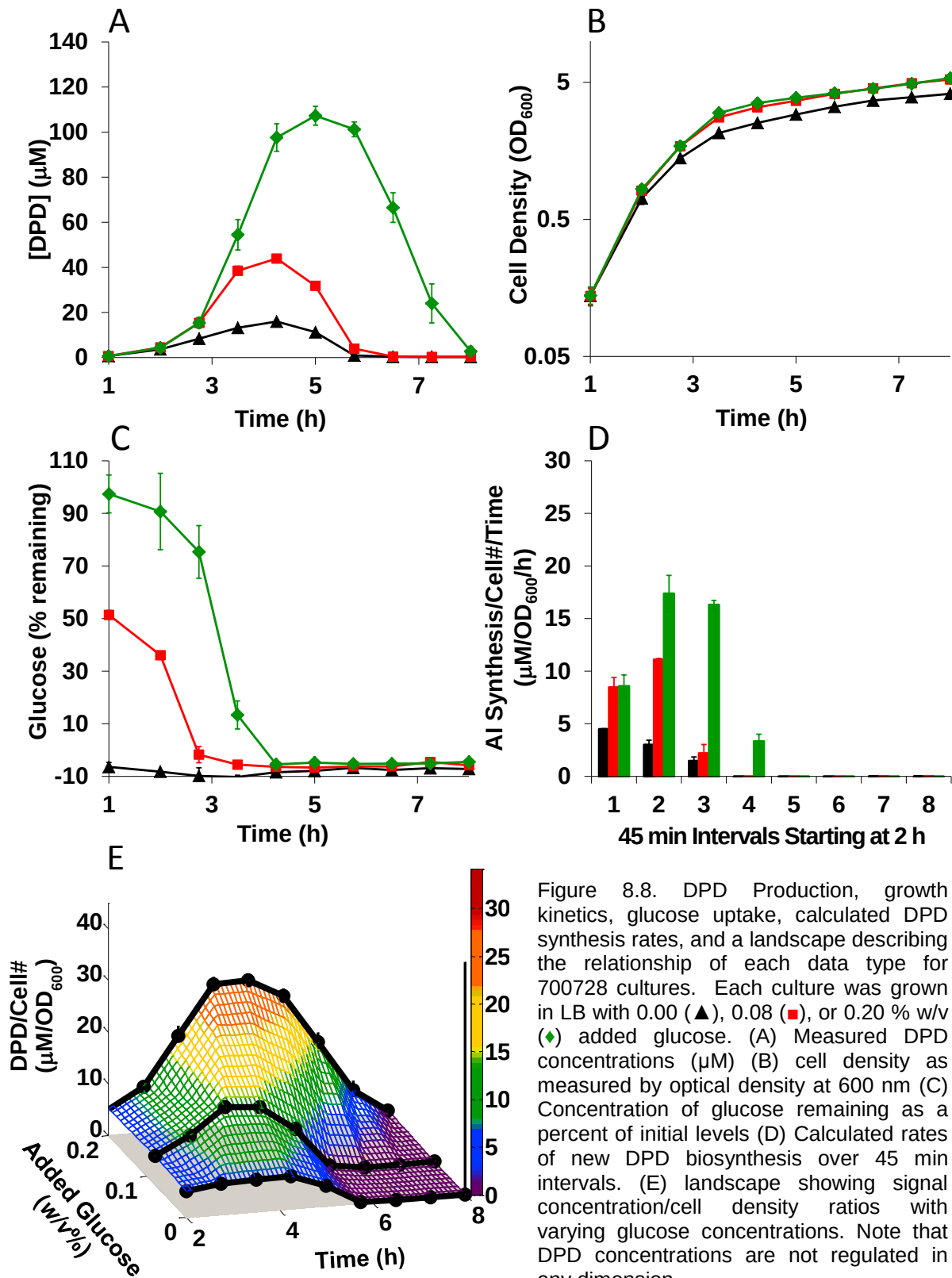


Figure 8.8. DPD Production, growth kinetics, glucose uptake, calculated DPD synthesis rates, and a landscape describing the relationship of each data type for 700728 cultures. Each culture was grown in LB with 0.00 ($\blacktriangle$), 0.08 ($\color{red}\blacksquare$), or 0.20 % w/v ($\color{green}\blacklozenge$) added glucose. (A) Measured DPD concentrations (μM) (B) cell density as measured by optical density at 600 nm (C) Concentration of glucose remaining as a percent of initial levels (D) Calculated rates of new DPD biosynthesis over 45 min intervals. (E) landscape showing signal concentration/cell density ratios with varying glucose concentrations. Note that DPD concentrations are not regulated in any dimension.

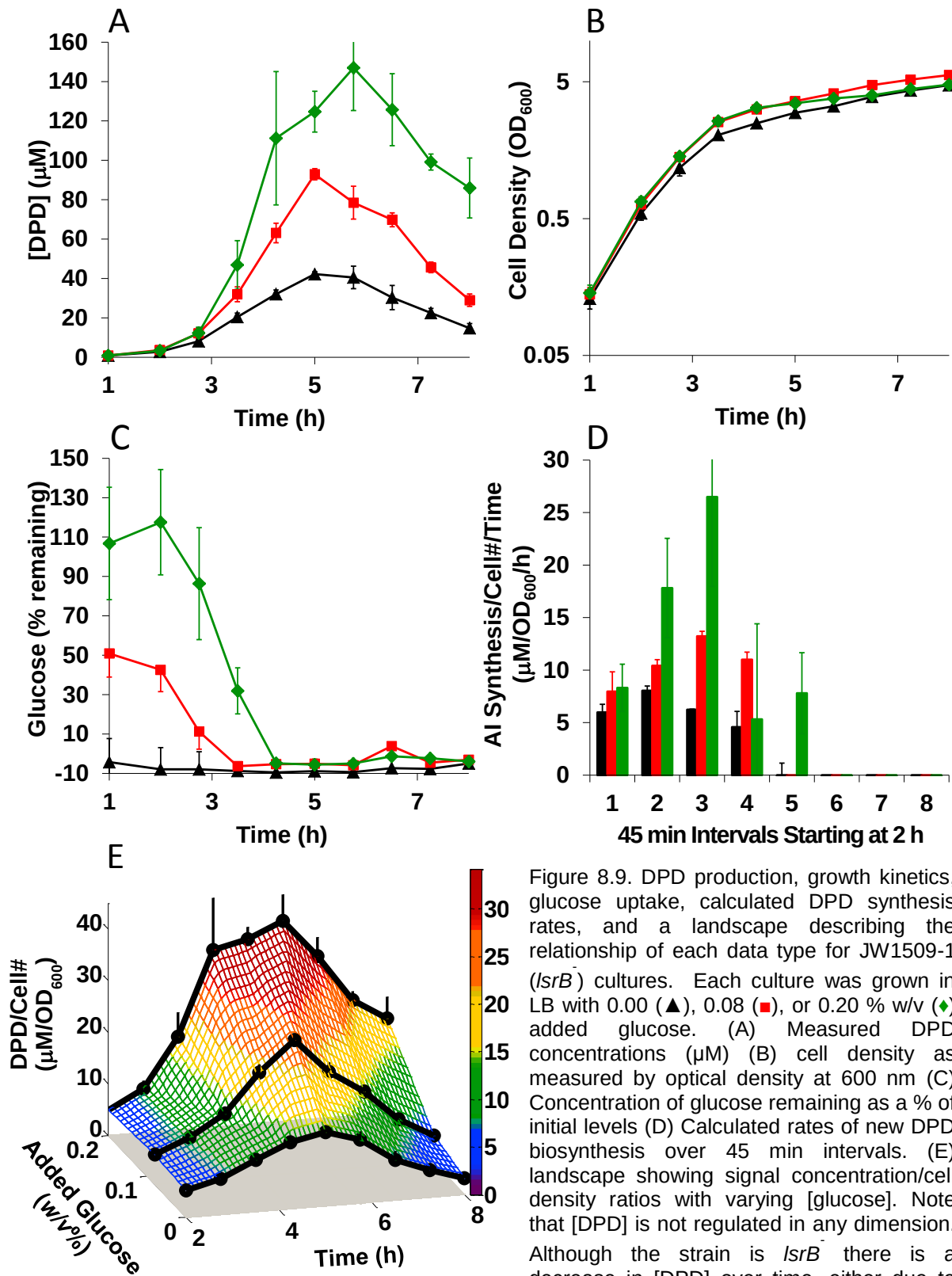


Figure 8.9. DPD production, growth kinetics, glucose uptake, calculated DPD synthesis rates, and a landscape describing the relationship of each data type for JW1509-1 (*IsrB*⁻) cultures. Each culture was grown in LB with 0.00 ($\blacktriangle$), 0.08 ($\color{red}\blacksquare$), or 0.20 % w/v ($\color{green}\blacklozenge$) added glucose. (A) Measured DPD concentrations (μM) (B) cell density as measured by optical density at 600 nm (C) Concentration of glucose remaining as a % of initial levels (D) Calculated rates of new DPD biosynthesis over 45 min intervals. (E) landscape showing signal concentration/cell density ratios with varying [glucose]. Note that [DPD] is not regulated in any dimension. Although the strain is *IsrB*⁻ there is a decrease in [DPD] over time, either due to degradation or an alternate importation mechanism.

mechanism at a slower rate than observed for the wild type strains with the exception of 25922. Contamination with an LsrB positive strain was ruled out because the *lsrB*⁻ cultures had been grown in the presence of kanamycin overnight.

In order to determine if the consumption of DPD might be due to degradation, a control experiment was run to determine the rate of DPD degradation in sterile media (Figure 8.10 panel A). DPD was added to 25 mL sterile LB at 57 μ M, incubated under the experimental conditions and monitored for 6.5 h. Over this time period only 7 μ M was lost. In contrast, *lsrB*⁻ *E. coli* cultures lost 27, 64, and 98 μ M in a 3 h period for 0.00, 0.08, and 0.20 w/v% added glucose cultures respectively. This indicates that either the bacteria are secreting unknown molecules capable of catalyzing DPD degradation, or another import mechanism is present. Considering the similarities noted between the Lsr transport complex and the ribose transporter^{173,204} as well as the similarities between AI-2 and other pentoses²⁰⁵ another low affinity transporter is a real possibility. We began work on a series of sugar transporter knockouts to study this mechanism, however early failures combined with more exciting leads discussed in Chapter 9 led to an executive decision to discontinue work in this area.

Also of concern in these experiments, was the observation of some acidification of the media at 0.2% w/v added glucose via color change in the derivatizing agent. We therefore tested all six *E. coli* strains in LB buffered to pH 7.05 with potassium phosphate to see if the pH played a role in DPD production rates (Figure 8.10 B-D). All cultures produced significantly less DPD under these conditions with most strains only reaching DPD concentrations in the 20-26 μ M range. While this experiment has not been repeated, it indicates that pH may play a major role in the regulation of DPD production. This tie between quorum sensing and the glucose acetate switch is not entirely novel. The HSL synthase AinS in *V. fischeri* regulates the switch from glucose to acetate as a carbon source.¹⁵¹

8.3 Regulation in Response to Exogenous DPD

We wondered if *V. harveyi* would attempt to regulate signal concentration in the presence of other sources of AI-2, and reasoned that exogenous DPD is likely to cause a regulatory perturbation. We anticipated one of three types of regulatory responses. 1) The bacteria could respond by increasing consumption or decreasing production of the

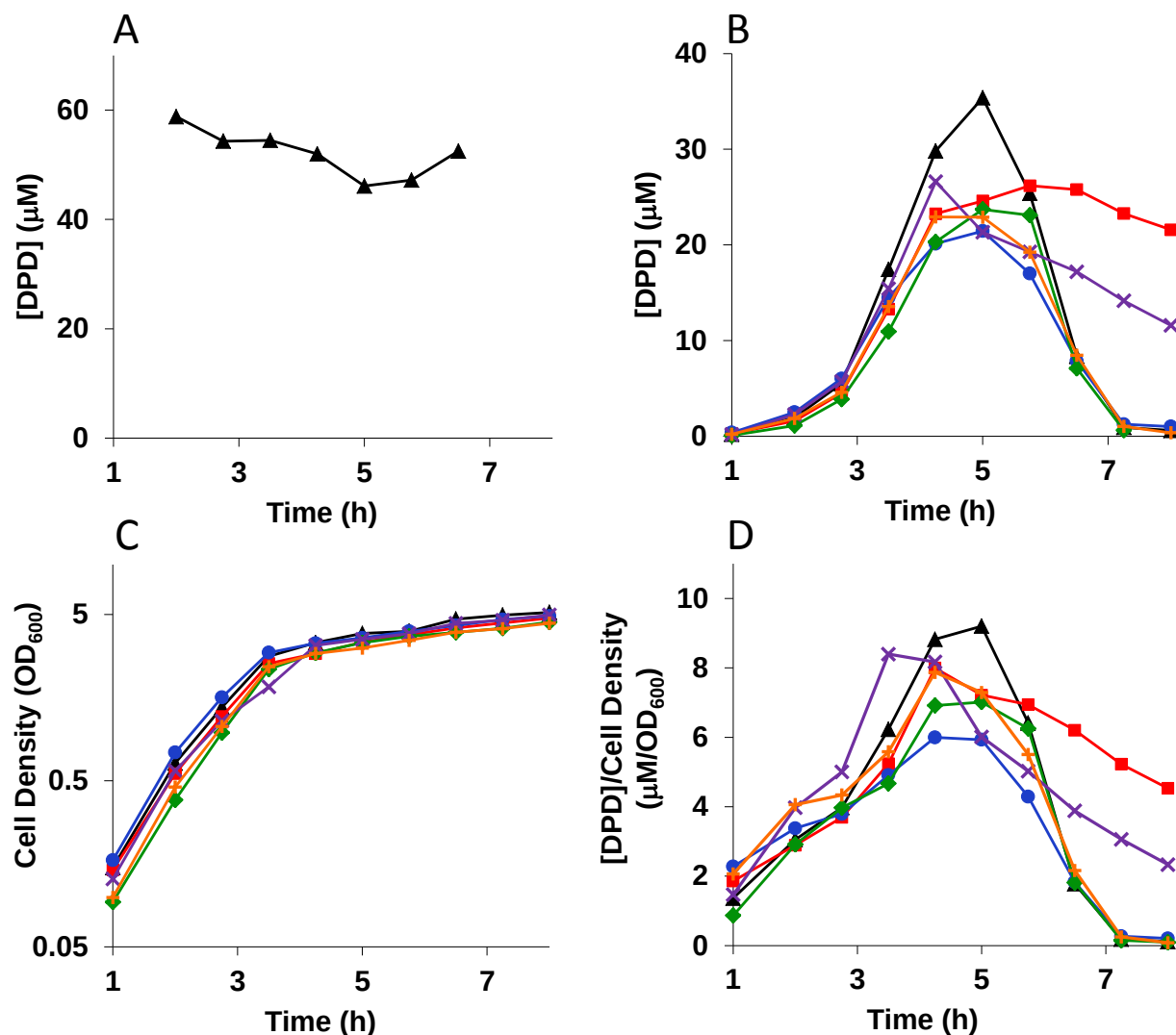


Figure 8.10. Control experiments for DPD degradation and pH effects. (A) Sterile LB was spiked with 56 μM DPD and the DPD concentration was measured over 6.5 h for signs of degradation. (B-D) DPD Production, growth kinetics, and plot of DPD/ OD_{600} for six *E. coli* strains: BW25113 ($\blacktriangle$), 8739 ($\bullet$), 25404 ($\blacklozenge$), 25922 ($\times$), 700728 ($+$), and JW1509-1 (*IsrB*⁻) ($\blacksquare$). Each culture was grown in pH 7.0 phosphate buffered LB with 0.20 % w/v added glucose. (B) Measured DPD concentrations (μM) (C) cell density as measured by optical density at 600 nm (D) DPD concentration/cell density ratios ($\mu\text{M}/\text{OD}_{600}$). These experiments have only been completed once. Note that DPD concentrations in panel B are dramatically different from other experiments indicating pH may play a role in regulation of DPD production.

DPD to lower the concentration to the expected levels. 2) The bacteria could continue to produce the same amount of DPD regardless of the external concentration, or 3) The bacteria could increase DPD production in response to the external signal. While both AIPs¹⁴⁴ and AHLs^{206,207} are known to positively feedback their own biosynthesis, AI-2 is not known to be regulated in this fashion. In fact, in species with the *lsr* operon, AI-2 is thought to upregulate its own consumption and degradation.²⁰⁸

Others have hypothesized that *E. coli* imports AI-2 solely as a defense mechanism to confuse quorum sensing signaling in invasive species.²⁰⁹ These experiments could also address this hypothesis by determining the extracellular DPD concentrations relevant to the induction of LsrB and therefore signal destruction.

To choose an appropriate time frame for the experiment, a culture of JW2662-1 was inoculated into fresh LB and grown for 5 h, a time point later than normal induction of the Lsr transport complex. This strain is *luxS*⁻, and therefore expression of the Lsr complex would not be induced prior to DPD treatment. At 5 h, the culture was spiked with various concentrations of DPD and [DPD] was measured every 15 min (Figure 8.11). All concentrations were found to induce *lsr* operon expression with the exception of 1 μ M DPD. Lsr transport proteins were obviously active within 30 min, and DPD was completely gone within 2 h. Therefore the 2 h time point was chosen for DPD addition since it is at a high enough cell density to observe a change and at least 30 min prior to normal induction of the Lsr transport complex.

For the following set of experiments, cultures were inoculated in fresh media, spiked with 0, 5, 10, 25, or 50 μ M DPD at the 2 h time point and sampled every 45 min for cell density and [DPD] (Figures 8.12-8.16). Where possible, the parent and *luxS* mutant strains were screened side by side to enable differentiation between exogenously added DPD and newly synthesized DPD.

V. harveyi cultures were only screened at 10 and 50 μ M DPD. These concentrations were chosen for being below and above the peak concentration in cultures grown in LM alone. We anticipated there may be a difference in regulatory response once a threshold concentration was reached. For the cultures to which 10 μ M DPD was added, no change in DPD production or consumption was observed. When the difference between the wild type (BB120) and *luxS* mutant (MM30) in the presence of DPD was plotted against the wild type with no added DPD the results were nearly identical (Figure 8.12 panel E). When 50 μ M DPD was added, similar results were obtained, until 5 h or the end of

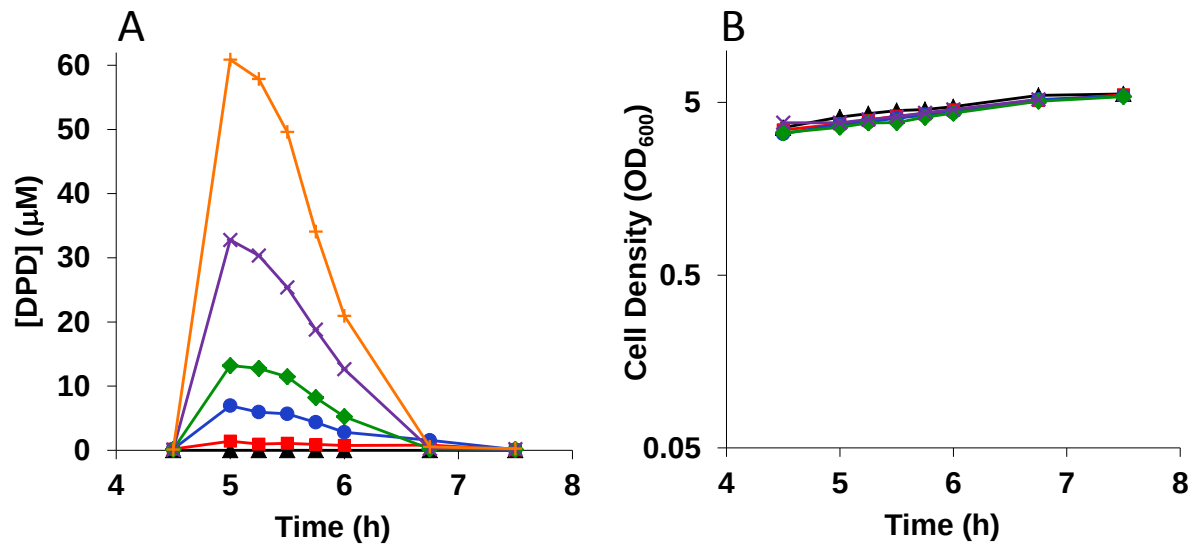


Figure 8.11. Pilot experiment to check the timing of LsrB induction. JW2662-1 (*luxS*⁻) cultures grown in LB and spiked with 0 ($\blacktriangle$), 1 ($\blacksquare$), 5 ($\bullet$), 10 ($\blacklozenge$), 25 ($\times$) or 50 ($+$) μM DPD at 5 h. (A) Measured DPD concentrations (μM). (B) Cell density as measured by optical density at 600 nm. Note that LsrB is induced within 30 min of the addition of DPD.

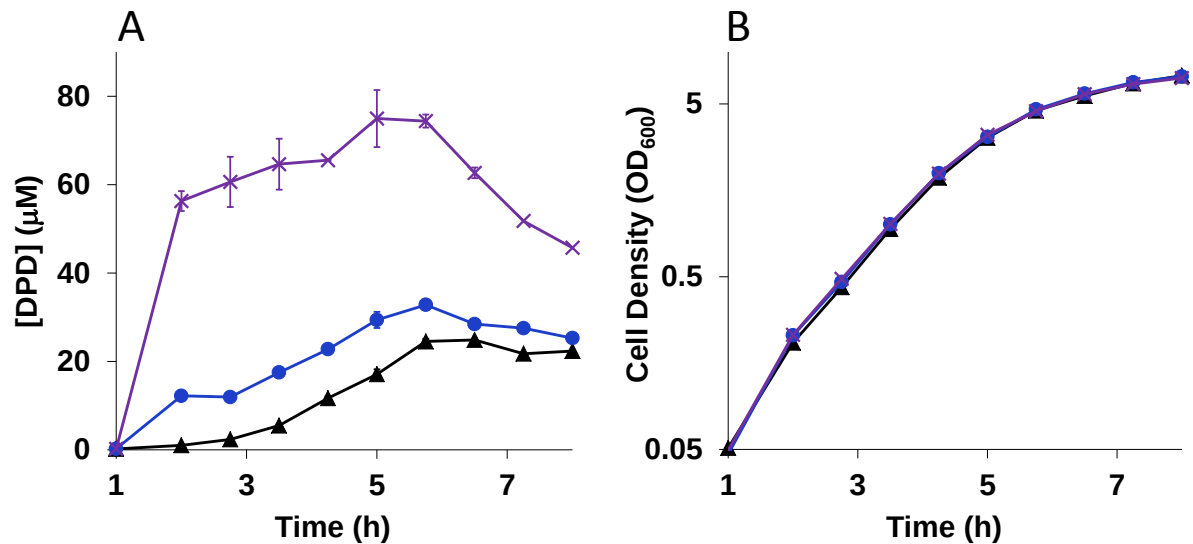


Figure 8.12. Response to extracellular DPD and growth kinetics in *V. harveyi* cultures grown in LM and spiked with 0 ($\blacktriangle$), 10 ($\bullet$), or 50 ($\times$) μM DPD at 2 h. Measured DPD concentrations (μM) in (A) BB120 and (C) MM30. Cell density as measured by optical density at 600 nm in (B) BB120 and (D) MM30. Overlay of the same data arranged by added DPD concentration: 10 (E) or 50 (F) μM . WT with no added DPD ($\blacktriangle$), WT + DPD ($\blacksquare$), *luxS*⁻ + DPD ($\bullet$), and WT + DPD - *luxS*⁻ + DPD ($\blacklozenge$).

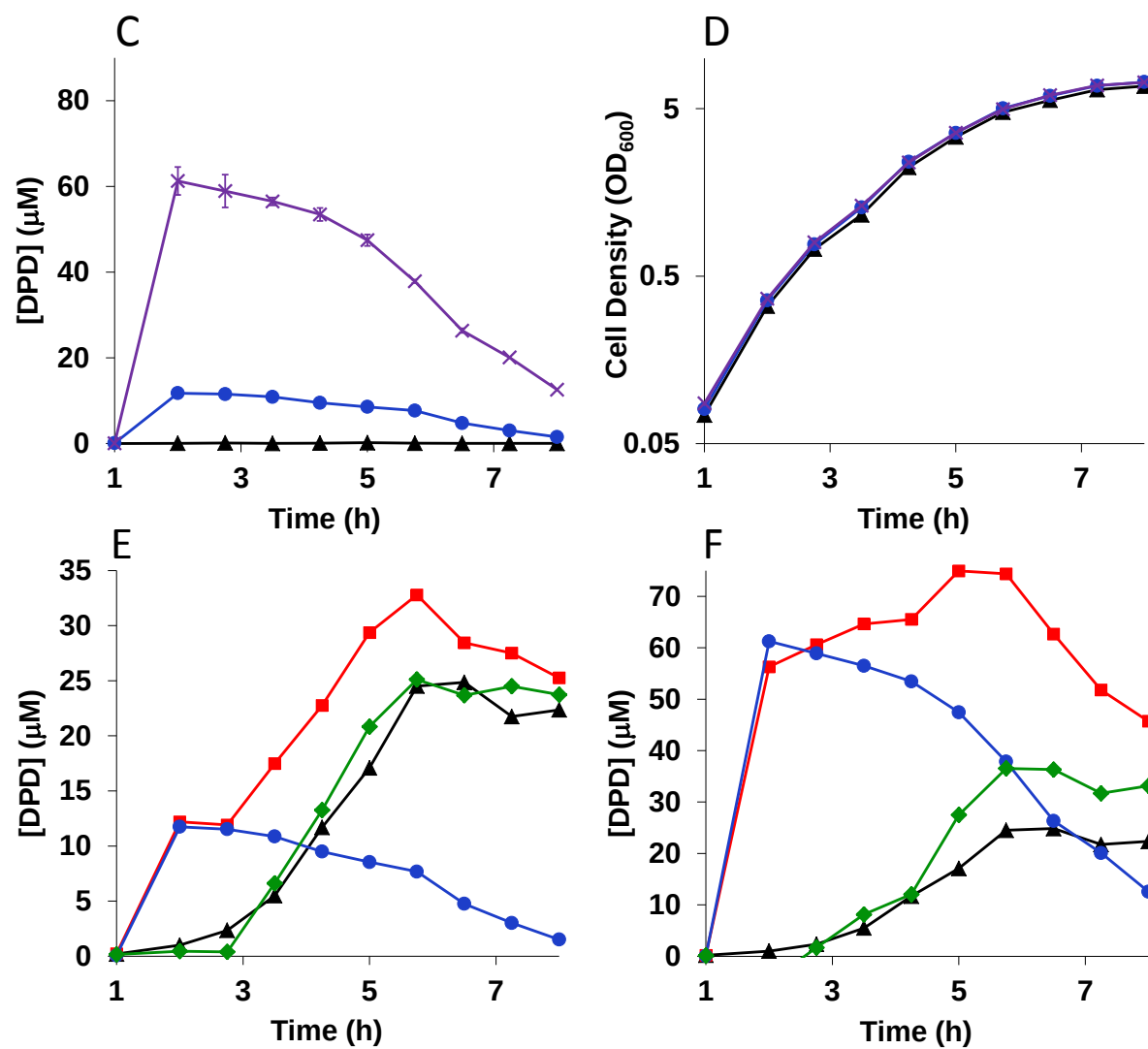


Figure 8.12. Continued.

exponential growth. After this time, approximately 10 μM of extra DPD was observed, and may be explained either by increased production in the wild type or increased consumption in the *luxS* mutant (Figure 8.12 panel F). The story remains however, that *V. harveyi* is capable of regulating its own production of DPD from low to high cell densities even in the presence of extracellular signal.

Turning next to *E. coli*, parent strain BW25113 and *luxS* mutant JW2662-1 did not show obvious signs of dysregulation upon inspection (Figure 8.13). When the difference between the treated parent and *luxS*⁻ strains were compared to the parent with no treatment, the data was almost identical with one exception (Figure 8.14). BW25113 treated with 50 μM DPD produced none of the expected DPD, and importation began at 4.25 h, 45 minutes earlier than usual. This demonstrates that *E. coli* can respond to extracellular DPD by upregulating LsrB production. Transcription of the *lsr* operon containing *lsrB* also expresses the DPD degradation proteins LsrF and LsrG, which could explain the absence of any observed production of DPD. Interestingly this response only occurs at concentrations of DPD twice that of *E. coli* production in laboratory conditions. While biologically relevant concentrations of DPD remain largely unknown, an influx of DPD of this magnitude seems unlikely in a foreign species invasion scenario. For this hypothesis to hold, the *E. coli* would have to remain in the high-cell density state with LsrB already induced and ready to degrade any extracellular AI-2.

The series of wild type *E. coli* strains were tested in an analogous fashion (Figure 8.15 and 8.16). However, since *luxS*⁻ strains were not available, we were unable to make comparisons or analyze the data as comprehensively. While the strains have characteristic DPD production curves, each did show a similar response pattern to BW25113, where importation of DPD begins earlier in response to 50 μM DPD and DPD production is inhibited. Moreover, strains 8739 and 700728 also show signs of early importation at 25 μM DPD, and signs of importation appear at 3.5 h in 8739.

As another control, each strain of *E. coli* was grown for 5 h to express the high density phenotype in LB and then diluted to an OD₆₀₀ typical of the 2 h time point in minimal salts, and 50 μM synthetic DPD was added. DPD was then measured in each culture every 30 min for 2 h (Figure 8.17). A pilot experiment was run with acetate added to some cultures to promote cell viability. There was little difference between the + and -

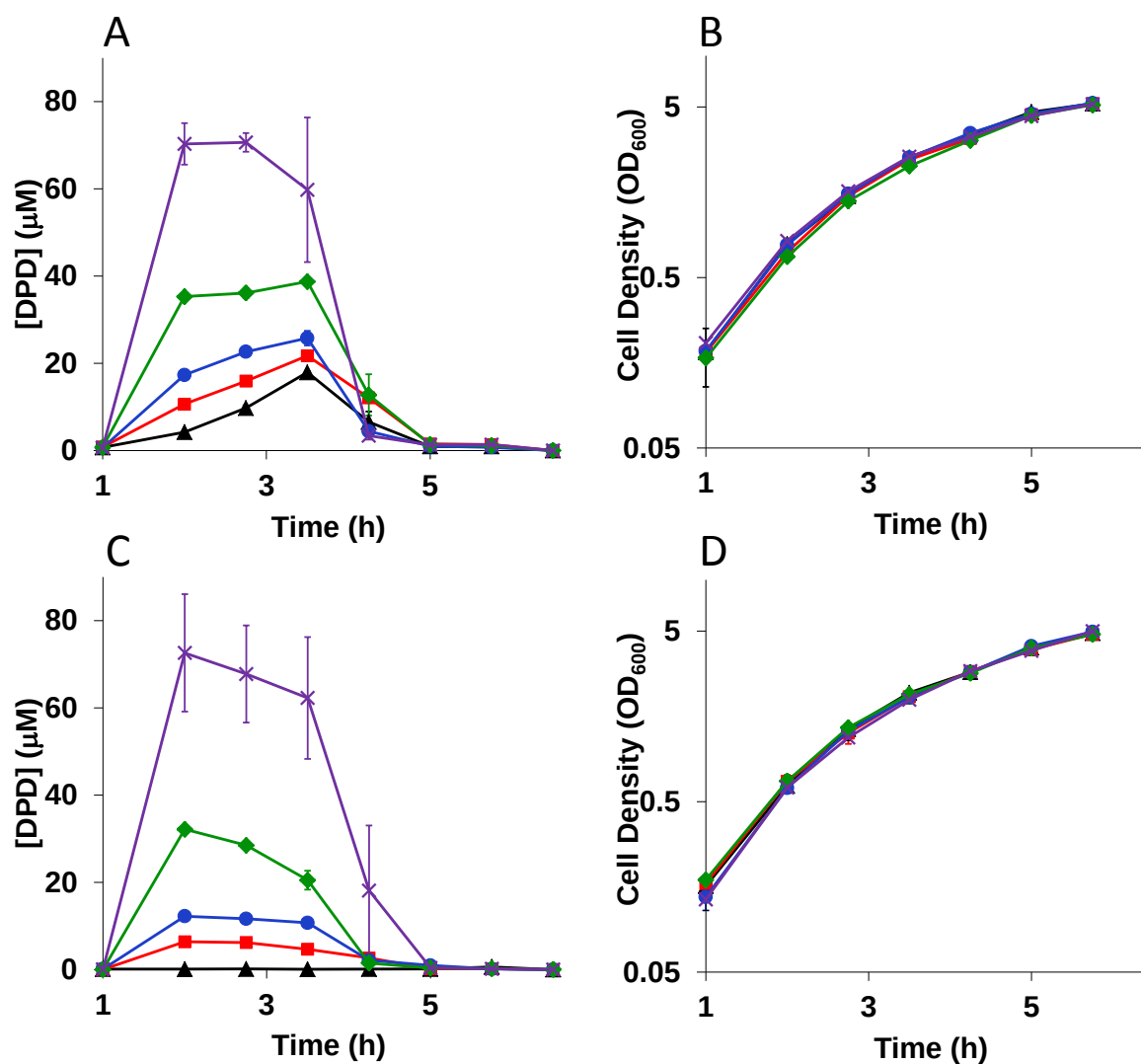


Figure 8.13. Response to extracellular DPD and growth kinetics in *E. coli* cultures grown in LB and spiked with 0 (▲), 5 (■), 10 (●), 25 (◆), or 50 (×) μM DPD at 2 h. Measured DPD concentrations (μM) in (A) BW25113 and (C) JW2662-1. Cell density as measured by optical density at 600 nm in (B) BW25113 and (D) JW2662-1.

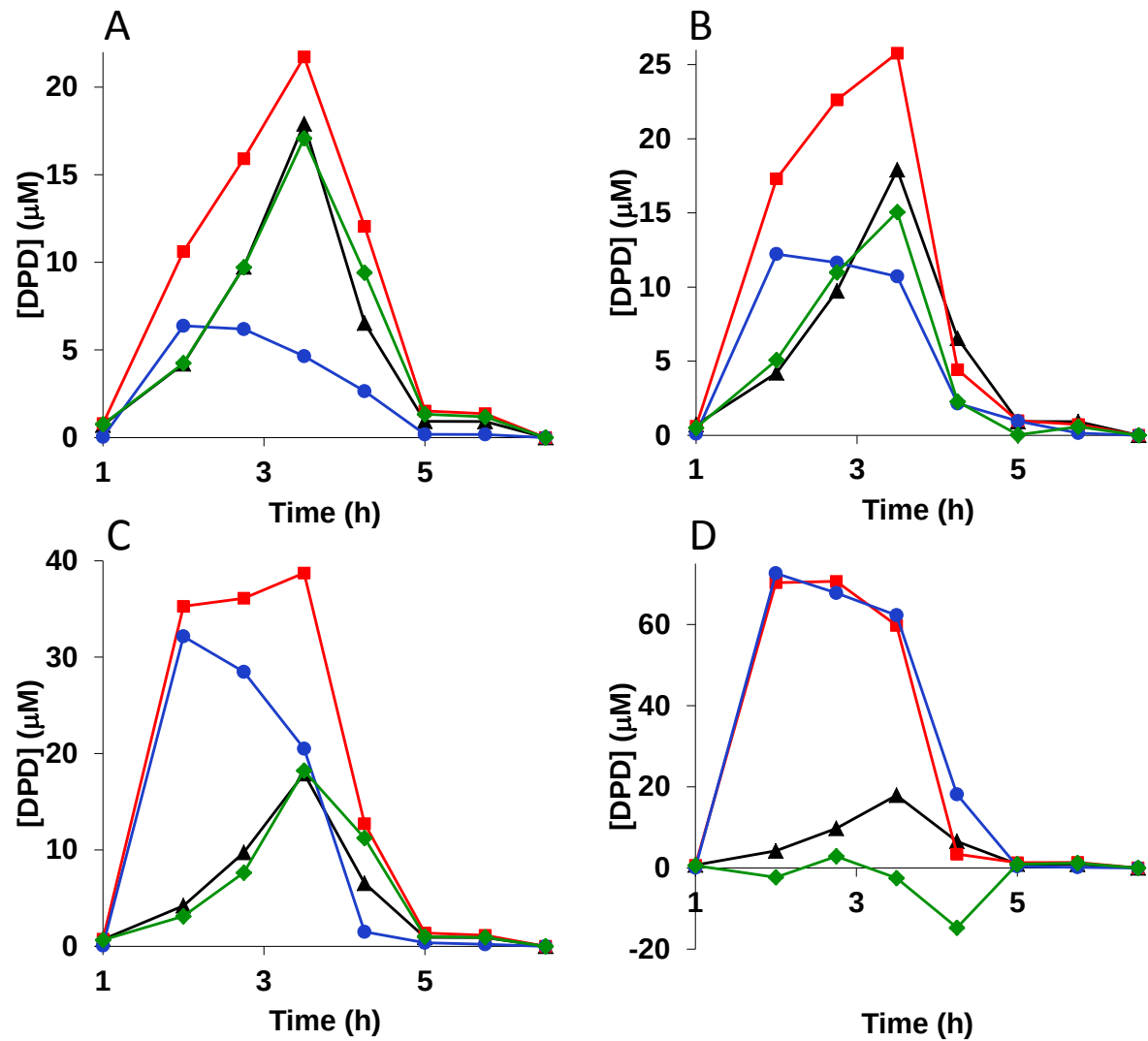


Figure 8.14. Response to extracellular DPD in *E. coli* cultures grown in LB and spiked with DPD at 2 h. This is the same data presented in Figure 8.13 arranged by added DPD concentration: 5 (A), 10 (B), 25 (C), or 50 (D) μM. WT with no added DPD (▲), WT + DPD (■), *luxS*⁻ + DPD (●), and WT + DPD - *luxS*⁻ + DPD (◆). Note that WT with no added DPD and WT + DPD - *luxS*⁻ + DPD are very similar at all concentrations but 50 μM.

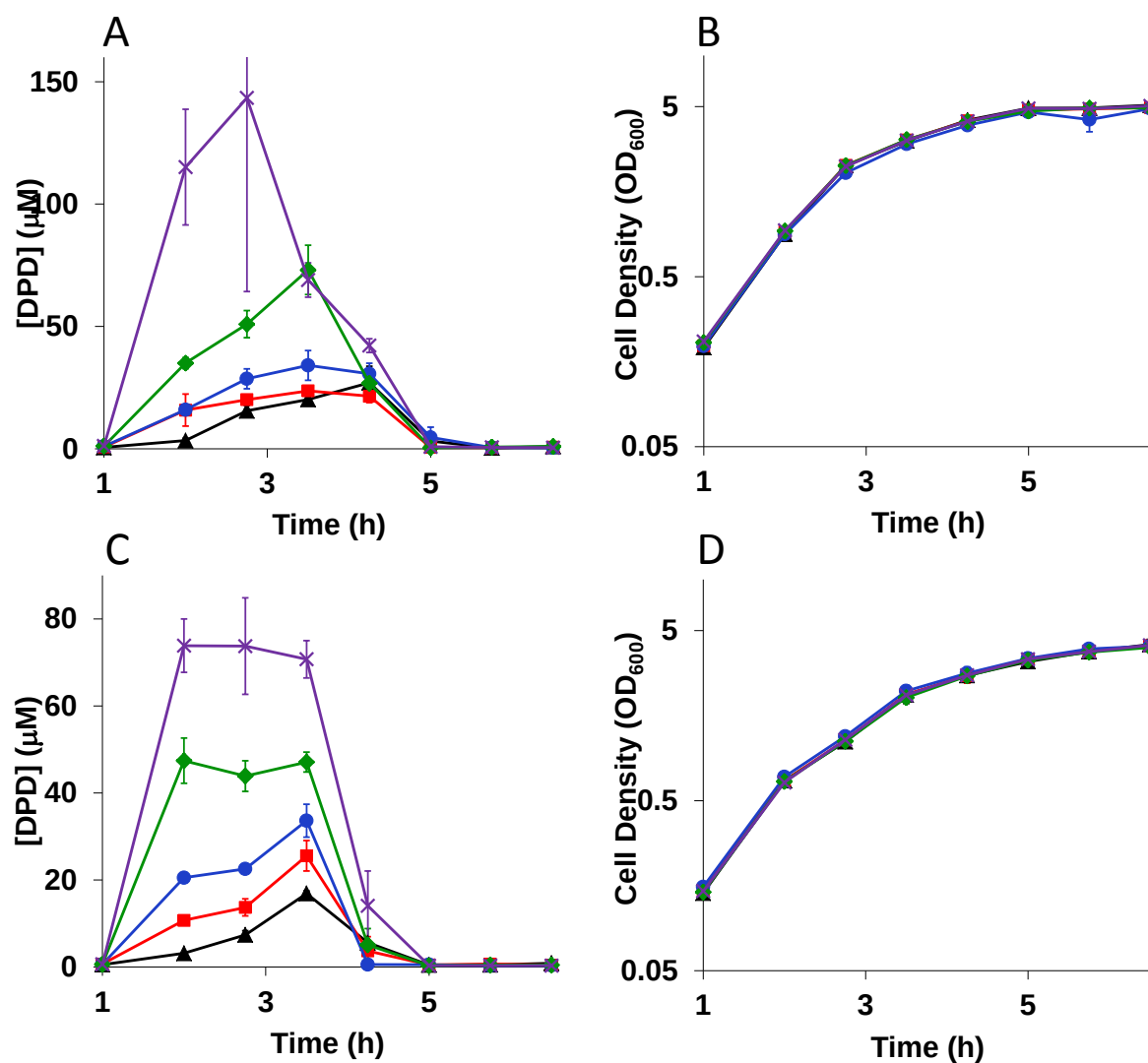


Figure 8.15. Response to extracellular DPD and growth kinetics in *E. coli* cultures grown in LB and spiked with 0 ($\blacktriangle$), 5 ($\blacksquare$), 10 ($\bullet$), 25 ($\blacklozenge$), or 50 ($\times$) μM DPD at 2 h. Measured DPD concentrations (μM) in (A) 8739 and (C) 25404. Cell density as measured by optical density at 600 nm in (B) 8739 and (D) 25404.

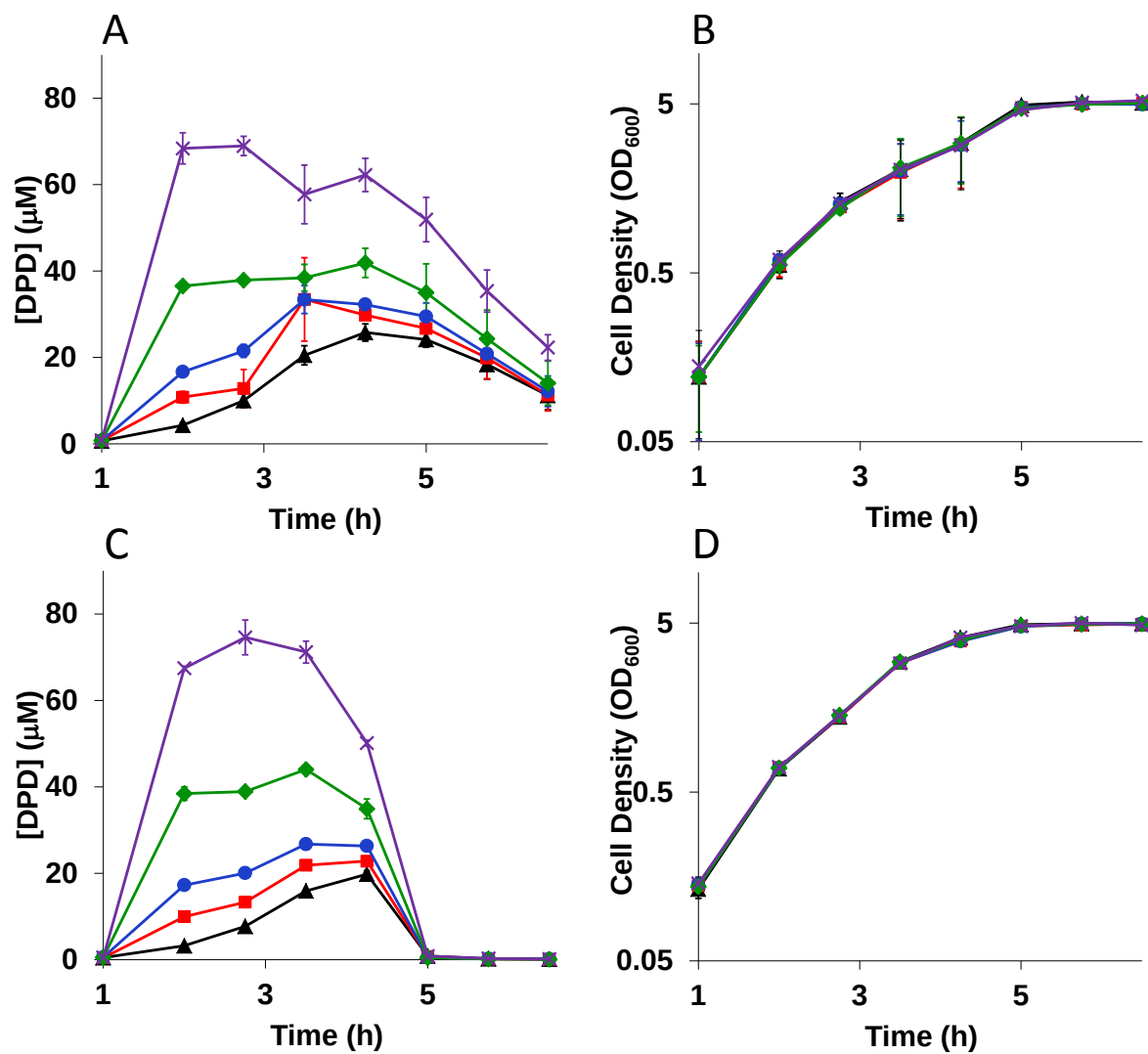


Figure 8.16. Response to extracellular DPD and growth kinetics in *E. coli* cultures grown in LB and spiked with 0 ($\blacktriangle$), 5 ($\color{red}\blacksquare$), 10 ($\bullet$), 25($\blacklozenge$), or 50 ($\times$) μM DPD at 2 h. Measured DPD concentrations (μM) in (A) 25922 and (C) 700728. Cell density as measured by optical density at 600 nm in (B) 25922 and (D) 700728.

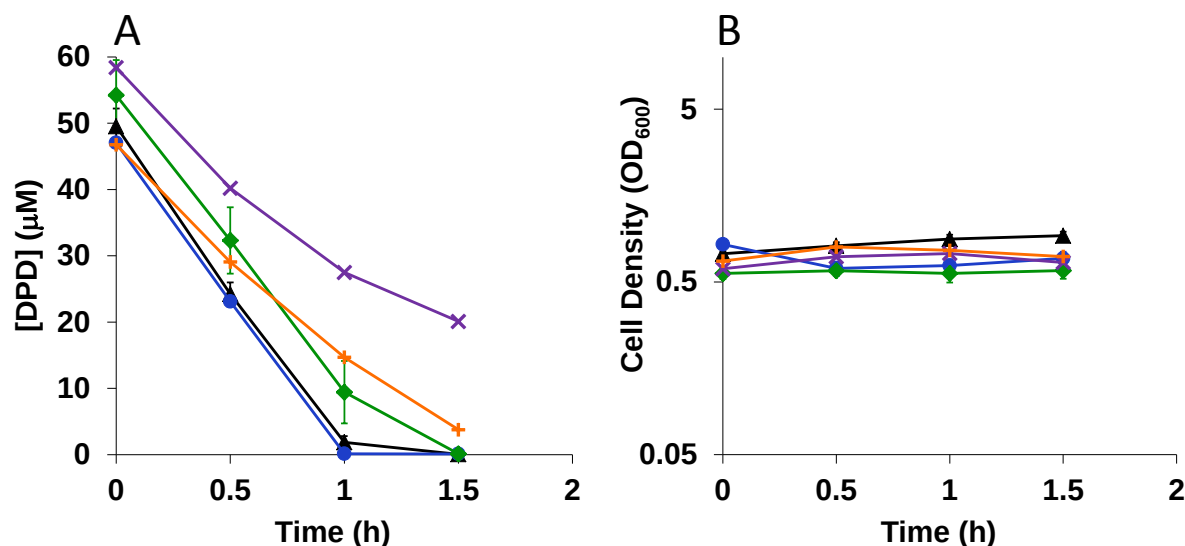


Figure 8.17. Cell density controls. *E. coli* cultures grown in LB for 5 h and inoculated to cell densities typical of 2 h in minimal salts containing 50 μ M DPD. (A) Measured DPD concentrations (μ M) (B) cell density as measured by optical density at 600 nm. Data is for strains BW25113 ($\blacktriangle$), 8739 ($\bullet$), 25404 ($\blacklozenge$), 25922 ($\times$), and 700728 ($+$). Note that all strains are capable of DPD consumption and/or degradation, although 25922 does so at a slower rate.

acetate cultures except for a small reduction in DPD uptake with acetate. Thus acetate addition was discontinued. We were able to show that all strains could consume DPD at low cell densities when inoculated immediately from a high-cell density phenotype culture. The fastest to consume DPD were BW25113 and 8739, followed by 25404 and 700728, and slowest, although at an observable rate, was 25922. Therefore we expect to observe high cell density phenotypes if present in a low cell density cultures.

In extracellular DPD response experiments using JW1509-1 (*IsrB*⁻) maintained in (50 μ g/mL) kanamycin, importation was again observed although at slower rates than wild type strains (Figure 8.18). The Keio collection, which contains BW25113, JW2662-1 and JW1509-1, is known to have a few issues with reverse mutations. Consequently, we decided to screen the remainder of the ABC transport complex mutants, *IsrA*⁻, *IsrC*⁻ and *IsrD*⁻ (Figures 8.19 and 8.20). These all had similar importation rates to the *IsrB*⁻ strain in the response experiments as well as in the cell density control experiments. Therefore we consider it a real phenomenon that *LsrA*, *LsrB*, *LsrC*, and *LsrD* mutants of *E. coli* can import or degrade DPD through another mechanism. Interestingly, the wild type strain

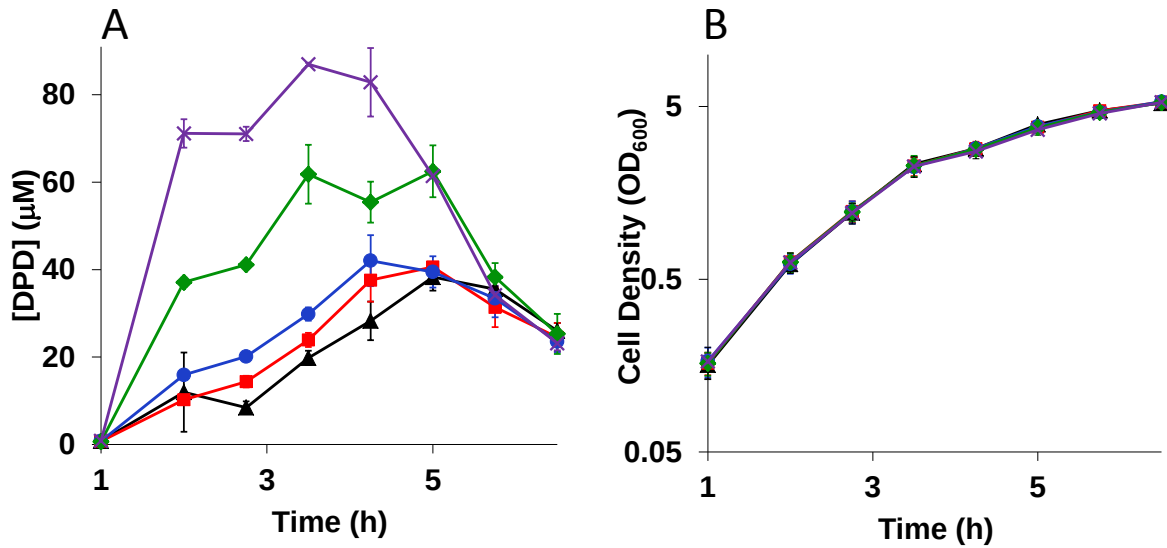


Figure 8.18. Response to extracellular DPD in JW1509-1 (*lsrB*⁻) grown in LB and spiked with 0 (▲), 5 (■), 10 (●), 25 (◆), or 50 (×) μM DPD at 2 h. (A) Measured DPD concentrations (μM). (B) Cell density as measured by optical density at 600 nm.

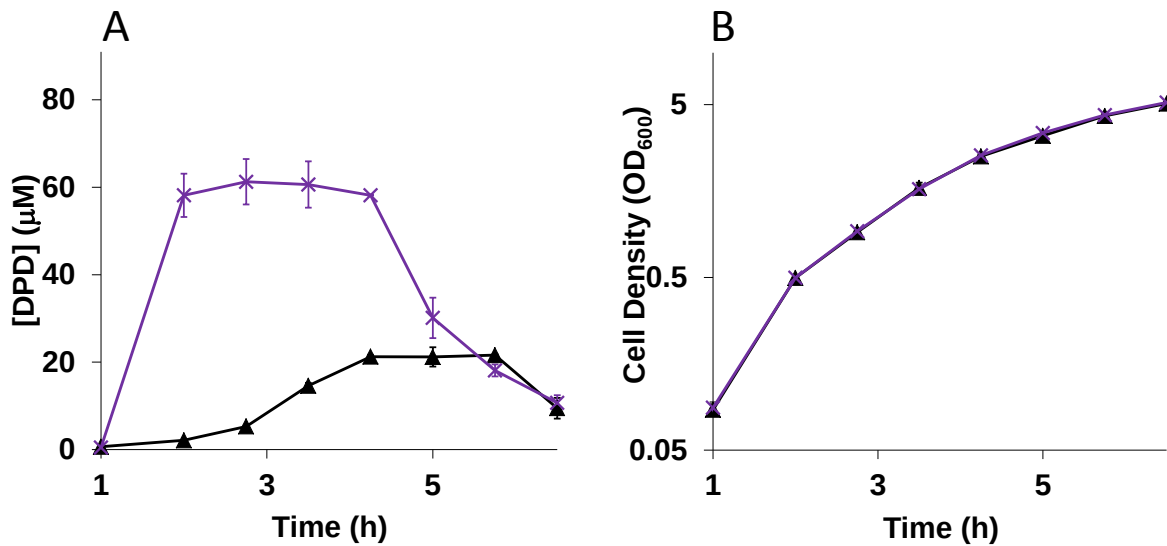


Figure 8.19. Response to extracellular DPD and growth kinetics in *E. coli* cultures grown in LB and spiked with 0 (▲) or 50 (×) μM DPD at 2 h. Measured DPD concentrations (μM) in (A) *lsrA*⁻ (C) *lsrC*⁻ and (E) *lsrD*⁻. Cell density as measured by optical density at 600 nm in (B) *lsrA*⁻ (D) *lsrC*⁻ and (F) *lsrD*⁻. Overlay of data from 25922 (□) showing similar importation rates in this strain compared to Lsr transport complex mutants.

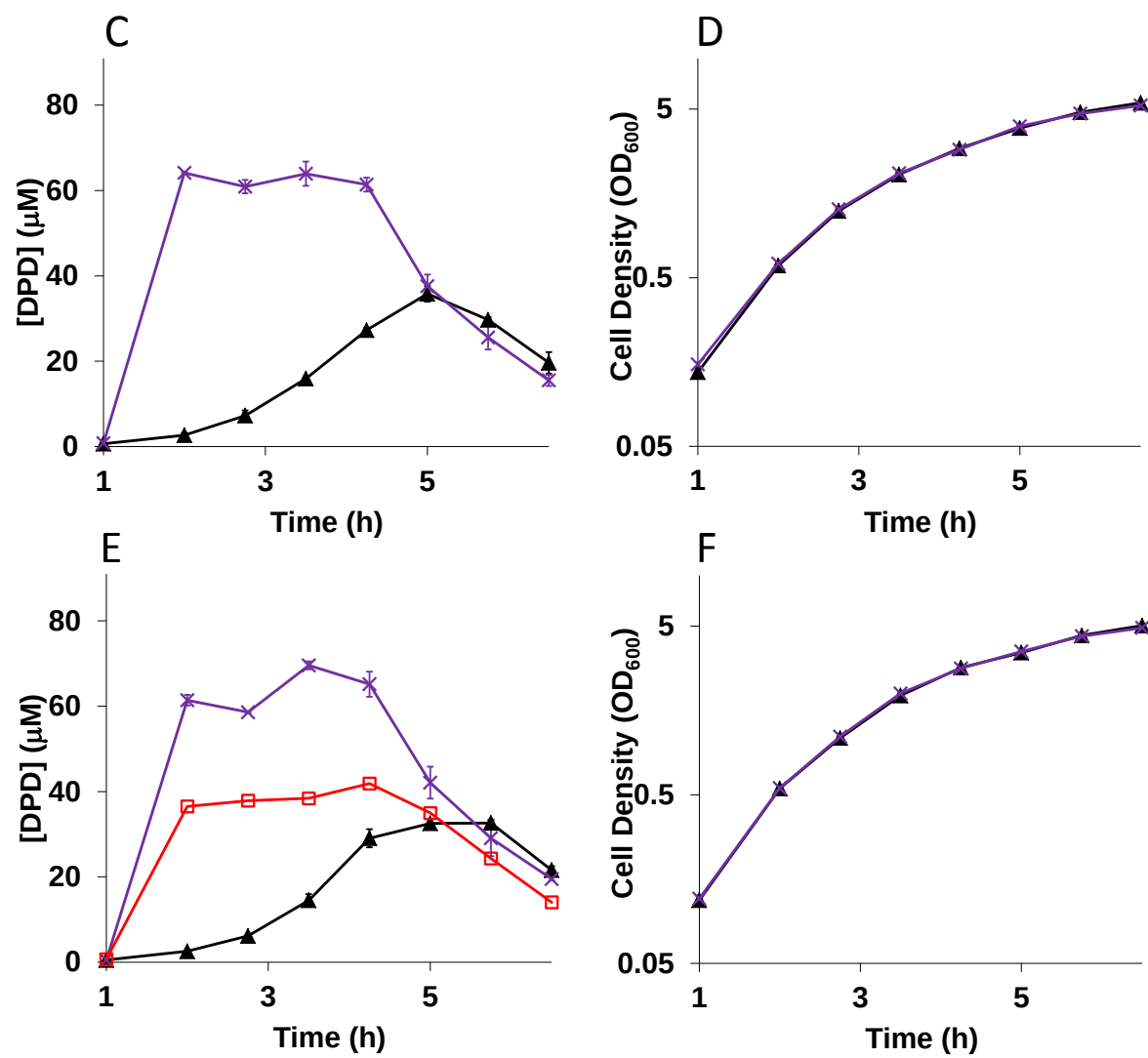


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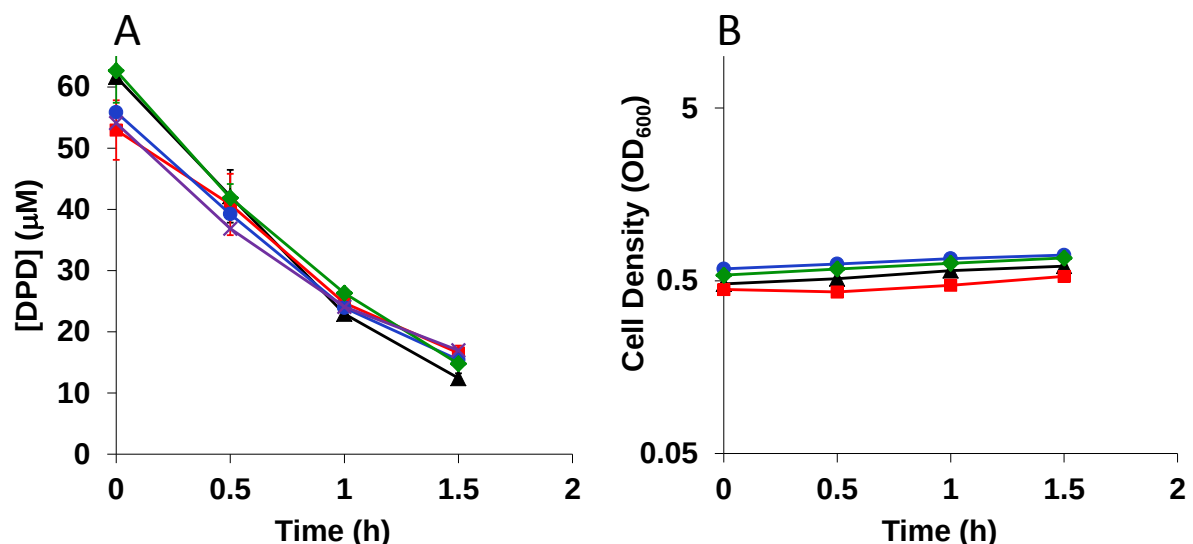


Figure 8.20. Cell density controls. *E. coli* mutants grown in LB for 5 h and inoculated to cell densities typical of 2 h in minimal salts containing 50 μM DPD. (A) Measured DPD concentrations (μM) (B) cell density as measured by optical density at 600 nm. Data is for strains *lsrA*⁻ ($\blacktriangle$), *lsrB*⁻ ($\blacksquare$), *lsrC*⁻ ($\bullet$), and *lsrD*⁻ ($\blacklozenge$). Overlay of data from 25922 ($\times$) showing similar importation rates in this strain compared to Lsr transport complex mutants. Note that all strains are capable of DPD consumption and/or degradation albeit slower than most wild type strains.

25922 has the same rate of importation when compared to Lsr transport complex mutants in both experiments. Hence, we propose that 25922 also has a defect impeding Lsr transport function.

8.4 A More Detailed Look at Biosynthesis

While the above experiments gave some insight into the nature of regulation in our two model species, we desired a more direct way of observing the biosynthesis of DPD. For this reason we developed a stable isotope flux profiling method capable of providing rate and synthetic pathway information and distinguishing between extracellular DPD and DPD synthesized de novo.

The biosynthetic source of the carbons in DPD can be traced to the ribose moiety of ATP. Therefore, if DPD is to be labeled by introduction of ($^{13}\text{C}_6$)glucose, the most direct routes are through the pentose phosphate pathway giving the common precursor,

ribulose-5-phosphate. This can occur through several pathways although four are most efficient. All 5 ^{13}C may be introduced at once through the conversion of glucose to gluconate-6-phosphate. Alternatively, ribulose-5-phosphate can be built from 2 and 3 carbon units through fructose-6-phosphate and glyceraldehyde-3-phosphate interconversions, respectively to give the DPD isotopomers shown (Figure 8.21). These isotopomers were chosen to be most relevant to the flux studies. However, more routes to labeled DPD are possible, although less likely, as other possible sugar interconversions available in bacteria could also be used.

In initial experiments, cultures of *V. harveyi* were spiked at 3 h of growth with 0.14% w/v ($^{13}\text{C}_6$)glucose, just after the exhaustion of unlabeled glucose from the rich media. The cultures were sampled and analyzed for 34 different SRMs. These SRMs consisted of all 21 possible SRMs based on 381-202 with up to 5 heavy isotopes incorporated into DPD-M1CQ. The remaining 13 included all possible SRMs for a DPD-M1CQ labeled with 5 heavy isotopes based on the other 3 most sensitive SRMs, 381-201, 381-231, and 381-363 (Table A8.1). No significant amounts of [$^{13}\text{C}_1$]DPD or [$^{13}\text{C}_4$]DPD were observed. Further, no isotopomer that would interfere with the quantitation of the (1- $^{13}\text{C}_1$)DPD internal standard was measured, thus allowing the addition of internal standard and exact quantitation in the final work.

Selection of the correct SRMs for the detection of the labeled isotopomers of DPD is critical for proper quantitation. The fragmentation of the molecule is not significantly affected by the inclusion of ^{13}C isotopes, but care must be taken to ensure that the proper reactions are monitored for the isotopomers as each reaction has differing efficiencies that could lead to quantitative errors if non-analogous reactions are compared. For DPD-M1CQ, the SRMs 381-201, 381-202, 381-231, and 381-363 are the most sensitive. This leads to 19 SRM options for the detection of ($^{13}\text{C}_5$)DPD-M1CQ (Figure 8.21 panel C). If all ^{13}C remain in the fragment, the appropriate SRMs are 386-206, 386-207, 386-236, and 386-368. However, if the fragment loses a ^{13}C , the corresponding SRMs will be 386-205, 386-206*, and 386-235, etc. The 386-368 SRM will remain unchanged as it corresponds to loss of water and cannot lose a ^{13}C . Each of the 19 possible SRMs for ($^{13}\text{C}_5$)DPD-M1CQ were monitored in cell-free supernatants to which (1- ^{13}C)DPD and M1CQ had been added, and the ratio of intensities for ($^{13}\text{C}_5$)DPD-M1CQ SRMs were then determined. For DPD-M1CQ the ratio of intensities was previously reported²¹⁰ as 22:43:47:100 for SRMs 381-201:381-202:381-231:381-363.

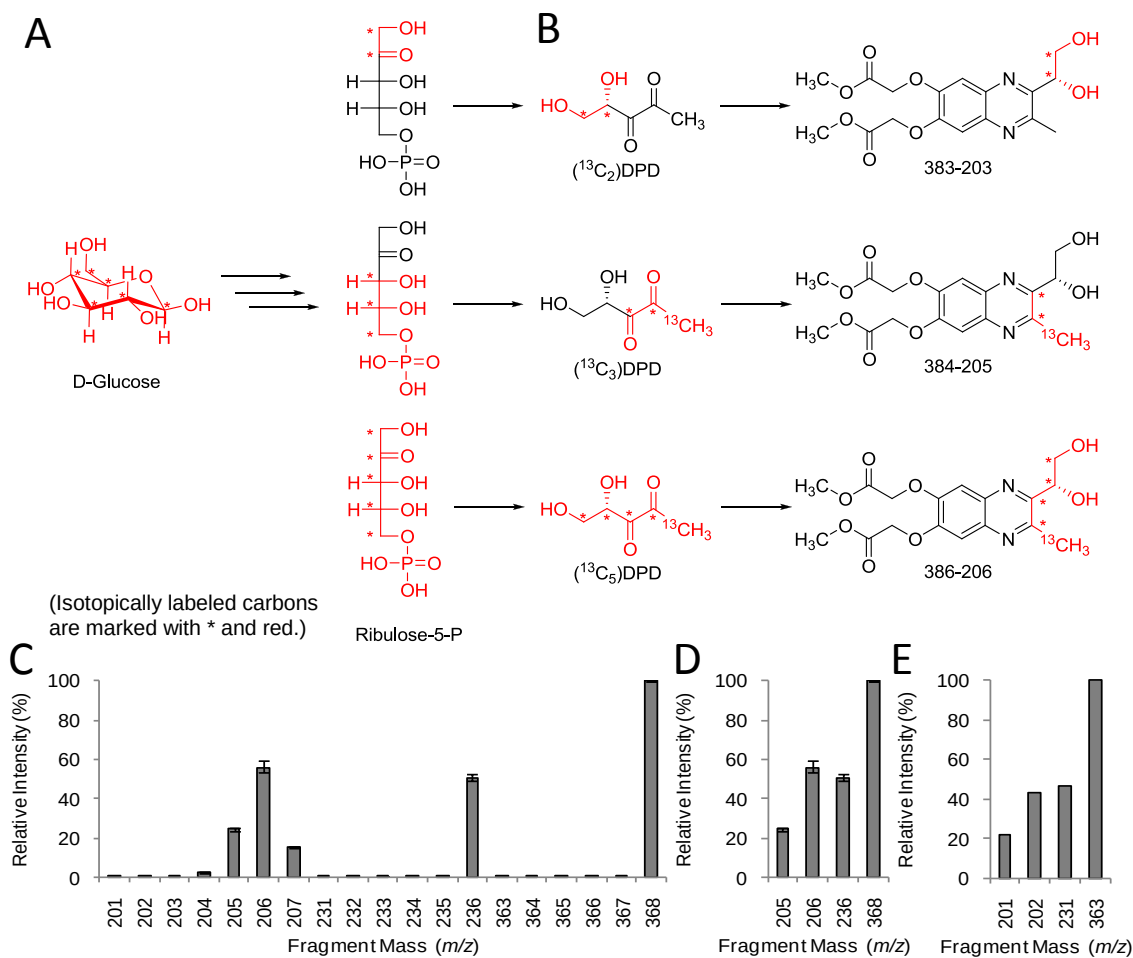


Figure 8.21. Selection of appropriate SRMs for flux experiments. (A) Biosynthetic routes to isotopically labeled ribulose-5P a common precursor to DPD. (B) Relevant isotopomers of DPD and their corresponding quinoxaline derivatives and SRMs listed as parent m/z – fragment m/z . (C) Relative intensities of all possible SRMs of $(^{13}\text{C}_5)\text{DPD-M1CQ}$. Reported numbers are the average of four experiments using cell-free supernatants of *V. harveyi* 120 min after addition 0.14% w/v $(^{13}\text{C}_6)\text{glucose}$ plus 28 mM DPD or 0.14% w/v $(^{13}\text{C}_6)\text{glucose}$ with no added DPD. Error bars express the range of the data. (D) and (E) graphs depicting analogous SRMs for $(^{13}\text{C}_5)\text{DPD-M1CQ}$ and DPD-M1CQ, respectively.

The ratio of the analogous reactions of ($^{13}\text{C}_5$)DPD-M1CQ should be similar to those for DPD-M1CQ. The measurement of the SRM intensity ratios for ($^{13}\text{C}_5$)DPD-M1CQ from *V. harveyi* supernatants was 25:56:51:100 for SRMs 386-205:386-206:386-236:386-368, respectively (Figure 8.21 panel D). The similarity of these two sets of ratios allowed us to choose an SRM for quantitation of ($^{13}\text{C}_5$)DPD-M1CQ, namely 386-206. This SRM corresponds to the SRM 381-202 that was used for quantitation of DPD-M1CQ in previous experiments. During fragmentation, ($^{13}\text{C}_5$)DPD-M1CQ loses a carbon from ($^{13}\text{C}_5$)DPD. From expected chemical reactivity, that carbon is almost certain to be C5. Also note that metabolic evidence²¹¹ indicates that ($^{13}\text{C}_2$)DPD is labeled at C4 and C5 and whereas ($^{13}\text{C}_3$)DPD is labeled at carbons 1-3. We also observed that ($^{13}\text{C}_2$)DPD loses a carbon during fragmentation but ($^{13}\text{C}_3$)DPD does not, again indicating that it is C5 that is lost during the fragmentation reaction.

With the analytical method established, *V. harveyi* strain BB120 and *E. coli* strains BW25113, 8739, 25404, 25922, and 700728 were profiled. Overnight cultures were inoculated into fresh rich media and spiked after exhaustion of unlabeled glucose (3 h for *V. harveyi* and 2.5 h for *E. coli*) with 0.04 or 0.14% w/v ($^{13}\text{C}_6$)glucose in the case of *V. harveyi* and *E. coli* *IsrB*⁻. For wild type *E. coli*, cultures were treated with 0.08 or 0.20% ($^{13}\text{C}_6$)glucose. The concentrations of DPD and its isotopologues were measured at intervals over 3 h using sampling methods reported in chapter 7 and then calculated using data from SRMs 381-202, 382-203, 383-203, 384-205, and 386-206.

We had previously observed regulation of extracellular DPD concentrations in *V. harveyi* and wished to discover whether this regulation was controlled at the biosynthesis level or by degradation of the excess signal in the medium. ($^{13}\text{C}_5$)DPD was observed within 15 min of ($^{13}\text{C}_6$)glucose addition and was produced preferentially until the depletion of glucose at 60 and 120 min for 0.04 and 0.14%, respectively (Figures 8.22 and 8.25 panel A). In the 0.04% glucose sample, we observed a switch back to an unlabeled carbon source after the 60 min time point. No decrease in levels of unlabeled DPD was observed, and total DPD concentrations reached 18 and 14 μM matching levels observed under similar conditions in previous experiments. This evidence supports control at a biosynthetic level for three reasons. First, production rates were maintained even when switching between carbon sources. Second, production rates are consistent with previous experiments in spite of a nutrient spike at 3 h. Finally, no

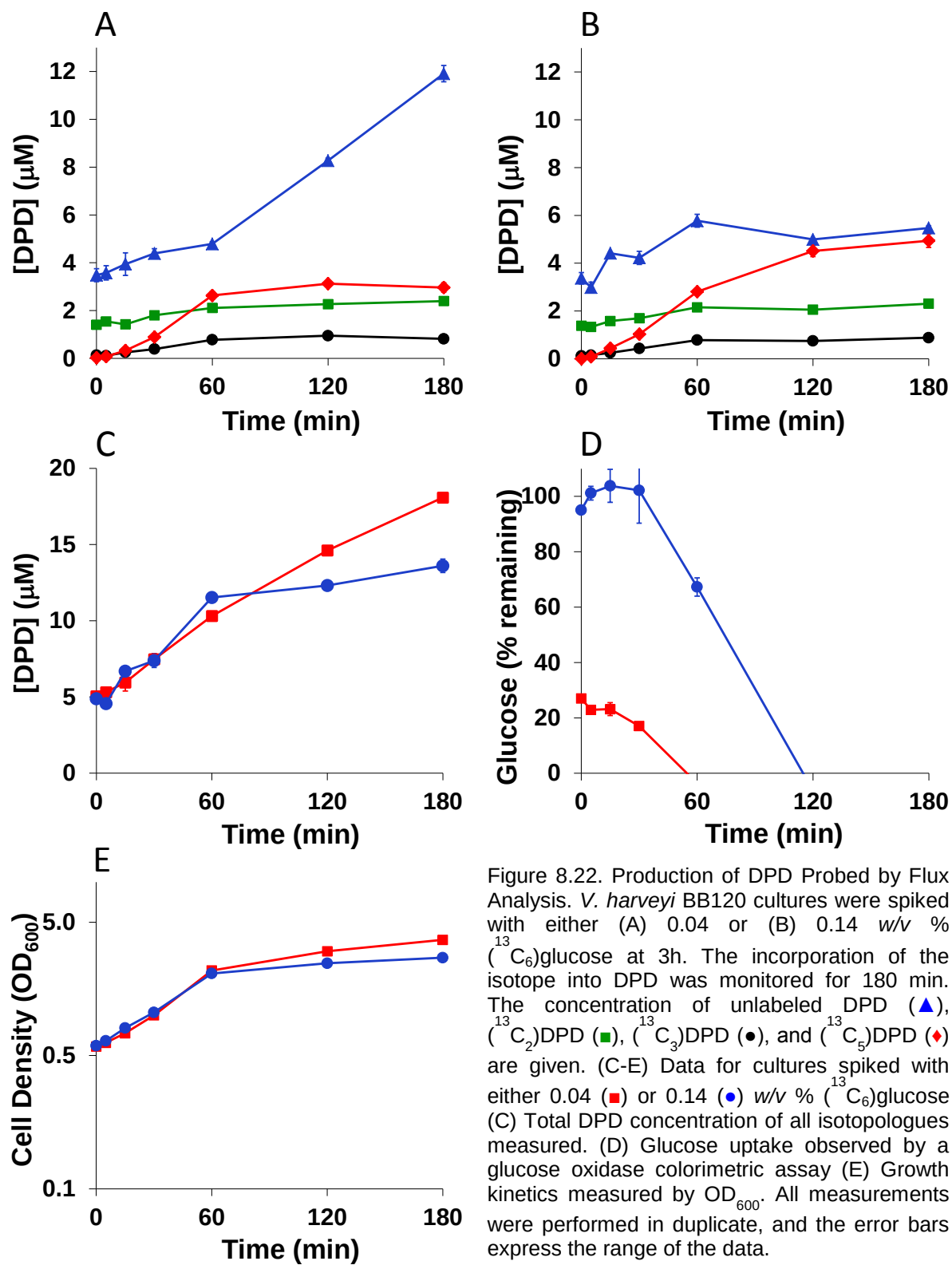


Figure 8.22. Production of DPD Probed by Flux Analysis. *V. harveyi* BB120 cultures were spiked with either (A) 0.04 or (B) 0.14 w/v % $^{13}\text{C}_6$ glucose at 3h. The incorporation of the isotope into DPD was monitored for 180 min. The concentration of unlabeled DPD ($\blacktriangle$), $^{13}\text{C}_2$ DPD ($\blacksquare$), $^{13}\text{C}_3$ DPD ($\bullet$), and $^{13}\text{C}_5$ DPD ($\blacklozenge$) are given. (C-E) Data for cultures spiked with either 0.04 ($\blacksquare$) or 0.14 ($\bullet$) w/v % $^{13}\text{C}_6$ glucose (C) Total DPD concentration of all isotopologues measured. (D) Glucose uptake observed by a glucose oxidase colorimetric assay (E) Growth kinetics measured by OD_{600} . All measurements were performed in duplicate, and the error bars express the range of the data.

degradation of signal was observed in this time frame thus rendering this mechanism of control unlikely.

We also wanted to validate our earlier findings that DPD synthesis in *V. harveyi* was not perturbed by an extracellular source of DPD. For these experiments *V. harveyi* cultures were spiked with 0.14% ($^{13}\text{C}_6$)glucose or 0.14% ($^{13}\text{C}_6$)glucose and 28 μM DPD (Figures 8.23 and 8.25 panel B). ($^{13}\text{C}_5$)DPD was observed within 30 min, and synthesis rates between the two treatments were identical. Again no appreciable signs of degradation were observed. This evidence validates our previous data and indicates that biosynthesis of DPD in *V. harveyi* is not disregulated by extracellular sources of DPD.

An *IsrB*⁻ mutant of *E. coli* BW25113 was screened first because it lacks the ability to remove extracellular DPD from the medium, allowing us to avoid complications due to degradation. ($^{13}\text{C}_5$)DPD was observed within 15 min of (U- ^{13}C)glucose addition and was made until the depletion of glucose at 60 and 120 min for 0.04% and 0.14% w/v ($^{13}\text{C}_6$)glucose, respectively (Figures 8.24 and 8.25 panel C). Production of unlabeled DPD continued during this time period at similar rates in both cultures. Experiments with these glucose concentrations had not previously been run to be able to compare the two, however these data follow the expected trends and demonstrate that the increase in DPD concentration with higher glucose is at least partially due to an upregulation of DPD biosynthesis.

Next, the series of wild type *E. coli* strains were profiled (Figures 8.26-8.31). For the 0.08% glucose samples in general, synthesis of ($^{13}\text{C}_5$)DPD was observed in 15 to 30 min and continued until 120 min. After glucose was depleted at 60 min an increase in unlabeled DPD synthesis rate was observed. Importation and degradation was observed beginning at 120 min. For the 0.20% glucose samples in general, synthesis of ($^{13}\text{C}_5$)DPD was observed in 15 to 30 min and continued until 60 min, at a rate identical to the 0.08% samples. Biosynthesis of ($^{13}\text{C}_5$)DPD continued preferentially until glucose was depleted at 120 min when it slowed, and an increase in unlabeled DPD synthesis was observed between 60 and 120 min. Signs of degradation were observed at 180 min in BW25113 and 700728 samples. Total concentrations of DPD fell between 27 and 40 μM for 0.08% samples and between 79 and 94 μM for 0.20% samples, which is lower than expected when compared to similar experimental conditions. However, this may be explained by the 2.5 h difference in addition of glucose. These experiments support the conclusion

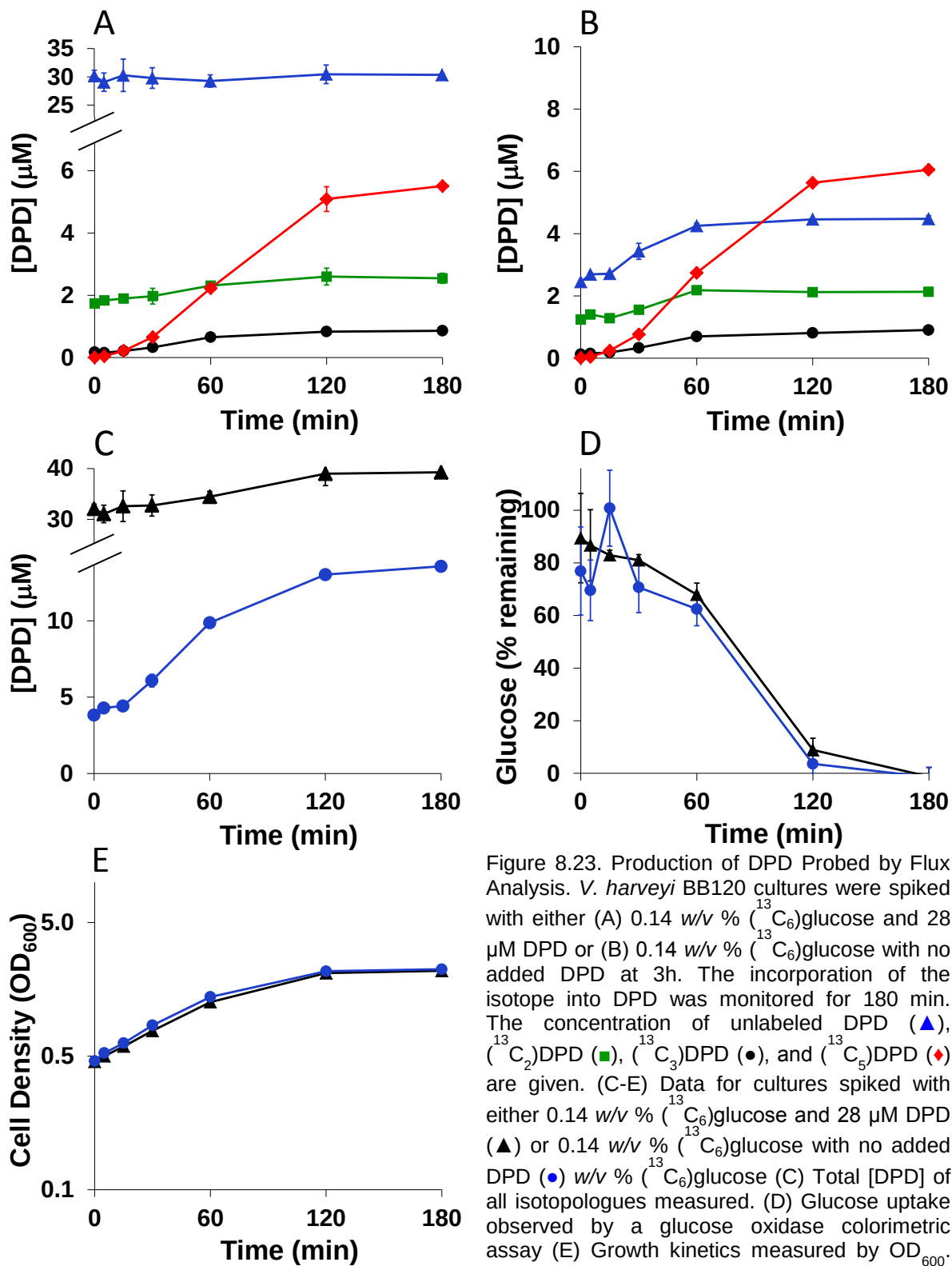


Figure 8.23. Production of DPD Probed by Flux Analysis. *V. harveyi* BB120 cultures were spiked with either (A) 0.14 w/v % $^{13}\text{C}_6$ glucose and 28 μM DPD or (B) 0.14 w/v % $^{13}\text{C}_6$ glucose with no added DPD at 3h. The incorporation of the isotope into DPD was monitored for 180 min. The concentration of unlabeled DPD (▲), $^{13}\text{C}_2$ DPD (■), $^{13}\text{C}_3$ DPD (●), and $^{13}\text{C}_5$ DPD (◆) are given. (C-E) Data for cultures spiked with either 0.14 w/v % $^{13}\text{C}_6$ glucose and 28 μM DPD (▲) or 0.14 w/v % $^{13}\text{C}_6$ glucose with no added DPD (●) w/v % $^{13}\text{C}_6$ glucose (C) Total [DPD] of all isotopologues measured. (D) Glucose uptake observed by a glucose oxidase colorimetric assay (E) Growth kinetics measured by OD₆₀₀. All measurements were performed in duplicate, and the error bars express the range of the data.

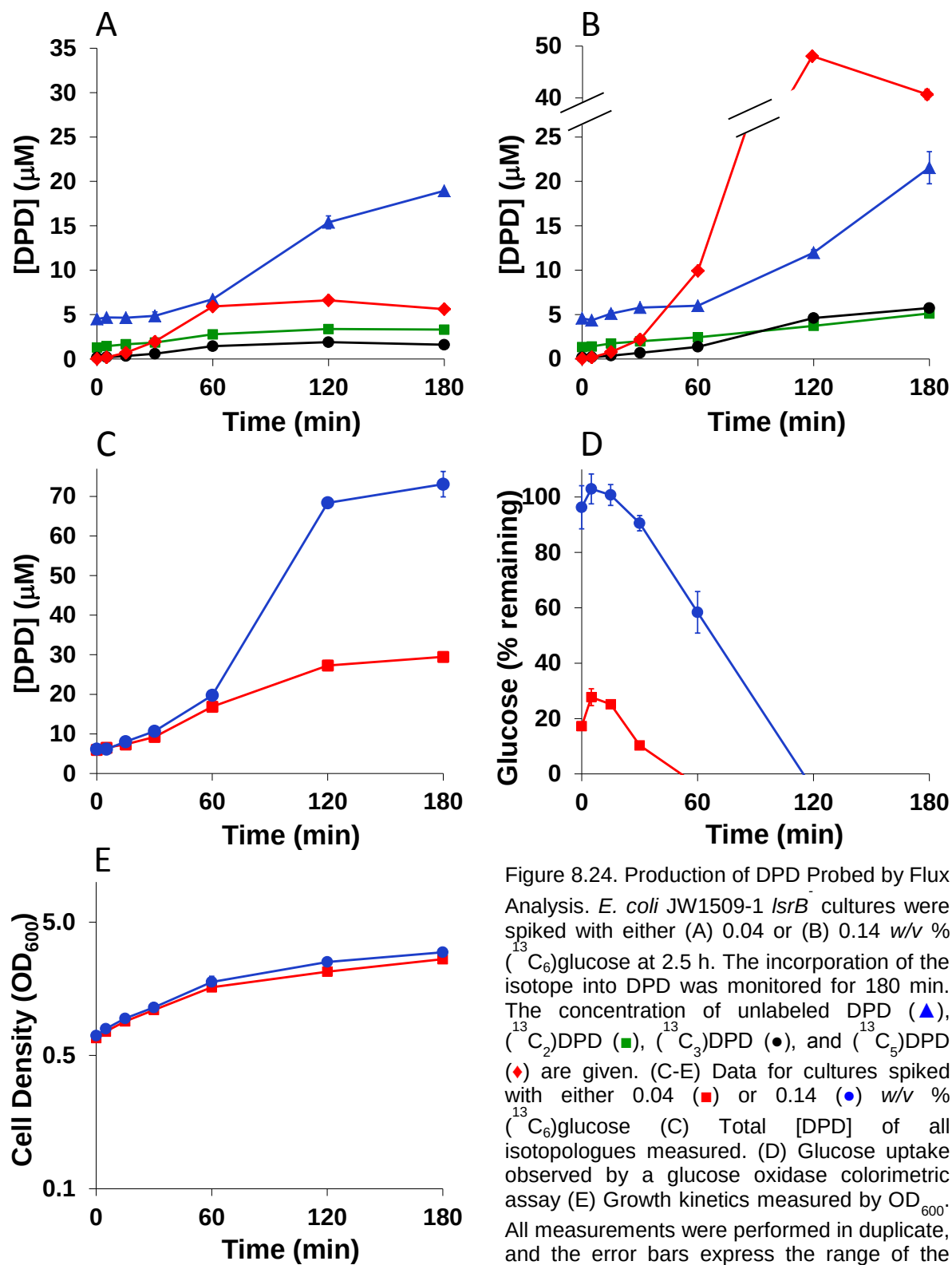


Figure 8.24. Production of DPD Probed by Flux Analysis. *E. coli* JW1509-1 ΔsrB cultures were spiked with either (A) 0.04 or (B) 0.14 w/v % ($^{13}\text{C}_6$)glucose at 2.5 h. The incorporation of the isotope into DPD was monitored for 180 min. The concentration of unlabeled DPD ($\blacktriangle$), ($^{13}\text{C}_2$)DPD ($\blacksquare$), ($^{13}\text{C}_3$)DPD ($\bullet$), and ($^{13}\text{C}_5$)DPD ($\blacklozenge$) are given. (C-E) Data for cultures spiked with either 0.04 ($\blacksquare$) or 0.14 ($\bullet$) w/v % ($^{13}\text{C}_6$)glucose (C) Total [DPD] of all isotopologues measured. (D) Glucose uptake observed by a glucose oxidase colorimetric assay (E) Growth kinetics measured by OD_{600} . All measurements were performed in duplicate, and the error bars express the range of the data.

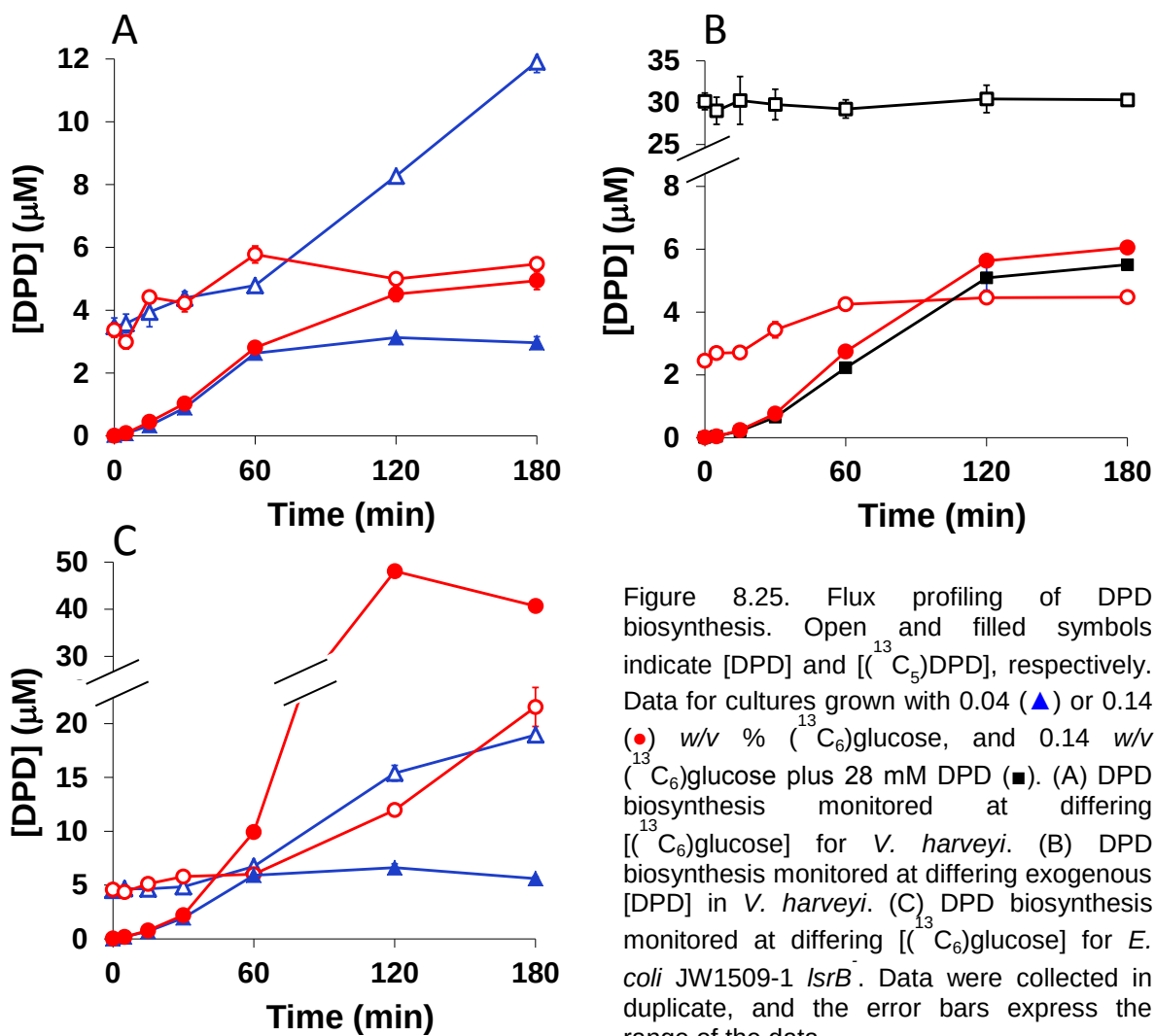


Figure 8.25. Flux profiling of DPD biosynthesis. Open and filled symbols indicate [DPD] and [$^{13}\text{C}_5$ DPD], respectively. Data for cultures grown with 0.04 ($\blacktriangle$) or 0.14 ($\bullet$) w/v % ($^{13}\text{C}_6$)glucose, and 0.14 w/v ($^{13}\text{C}_6$)glucose plus 28 mM DPD ($\blacksquare$). (A) DPD biosynthesis monitored at differing [$^{13}\text{C}_6$]glucose for *V. harveyi*. (B) DPD biosynthesis monitored at differing exogenous [DPD] in *V. harveyi*. (C) DPD biosynthesis monitored at differing [$^{13}\text{C}_6$]glucose for *E. coli* JW1509-1 *lsrB*⁻. Data were collected in duplicate, and the error bars express the range of the data.

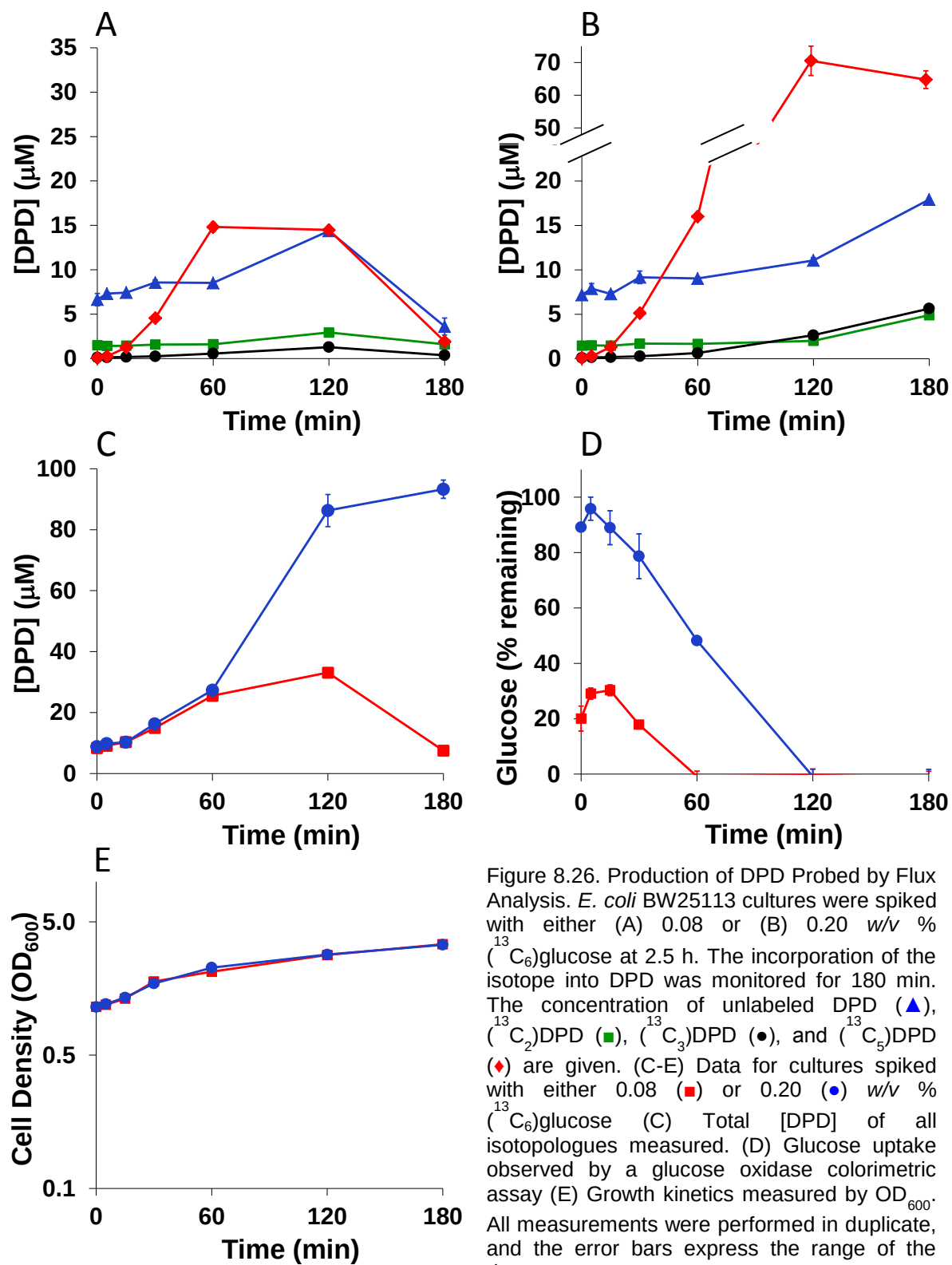


Figure 8.26. Production of DPD Probed by Flux Analysis. *E. coli* BW25113 cultures were spiked with either (A) 0.08 or (B) 0.20 w/v % ($^{13}\text{C}_6$)glucose at 2.5 h. The incorporation of the isotope into DPD was monitored for 180 min. The concentration of unlabeled DPD ($\blacktriangle$), ($^{13}\text{C}_2$)DPD ($\blacksquare$), ($^{13}\text{C}_3$)DPD ($\bullet$), and ($^{13}\text{C}_5$)DPD ($\blacklozenge$) are given. (C-E) Data for cultures spiked with either 0.08 ($\blacksquare$) or 0.20 ($\bullet$) w/v % ($^{13}\text{C}_6$)glucose (C) Total [DPD] of all isotopologues measured. (D) Glucose uptake observed by a glucose oxidase colorimetric assay (E) Growth kinetics measured by OD_{600} . All measurements were performed in duplicate, and the error bars express the range of the data.

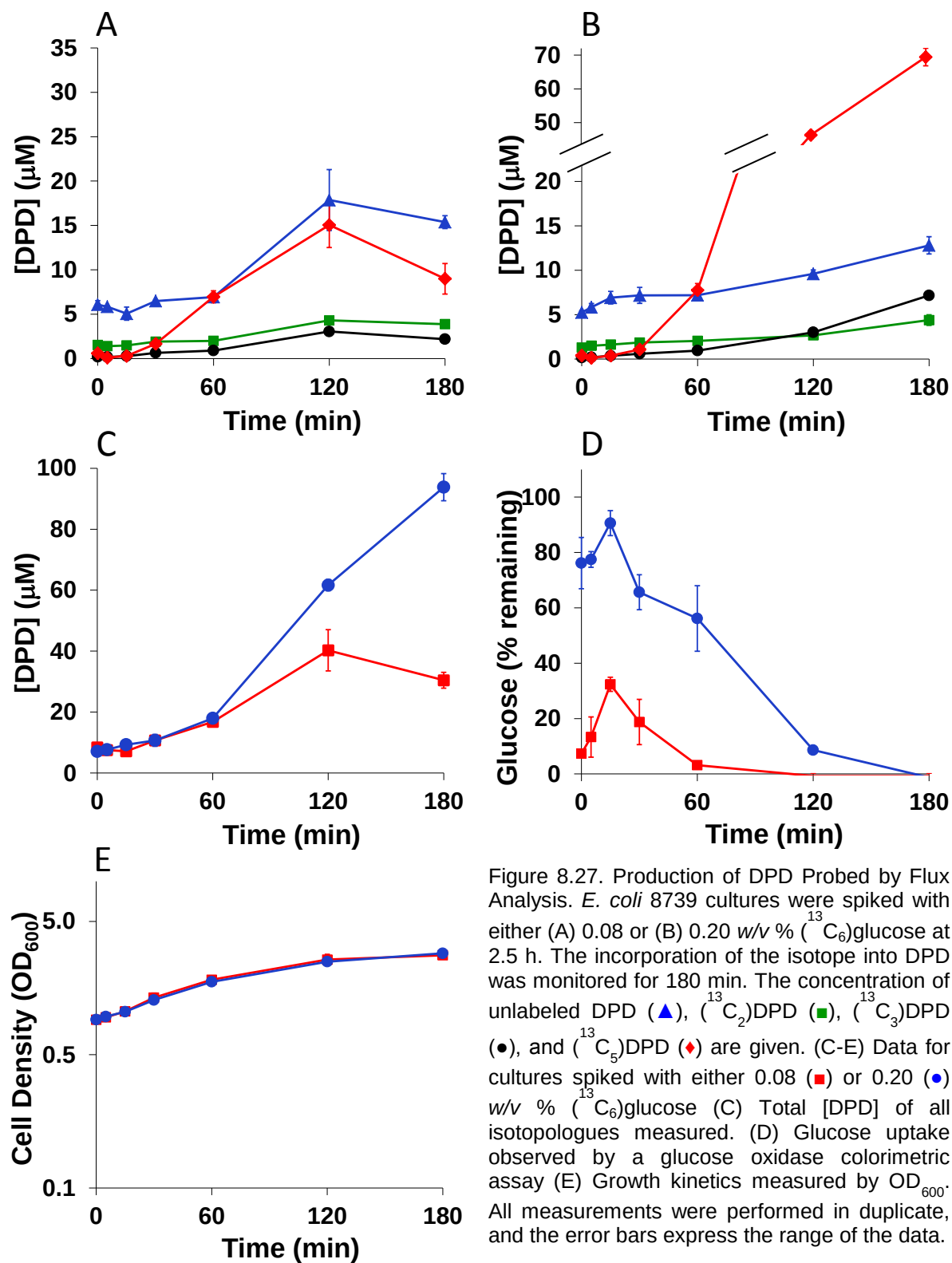


Figure 8.27. Production of DPD Probed by Flux Analysis. *E. coli* 8739 cultures were spiked with either (A) 0.08 or (B) 0.20 w/v % ($^{13}\text{C}_6$) glucose at 2.5 h. The incorporation of the isotope into DPD was monitored for 180 min. The concentration of unlabeled DPD ($\blacktriangle$), ($^{13}\text{C}_2$)DPD ($\blacksquare$), ($^{13}\text{C}_3$)DPD ($\bullet$), and ($^{13}\text{C}_5$)DPD ($\blacklozenge$) are given. (C-E) Data for cultures spiked with either 0.08 ($\blacksquare$) or 0.20 ($\bullet$) w/v % ($^{13}\text{C}_6$) glucose (C) Total [DPD] of all isotopologues measured. (D) Glucose uptake observed by a glucose oxidase colorimetric assay (E) Growth kinetics measured by OD₆₀₀. All measurements were performed in duplicate, and the error bars express the range of the data.

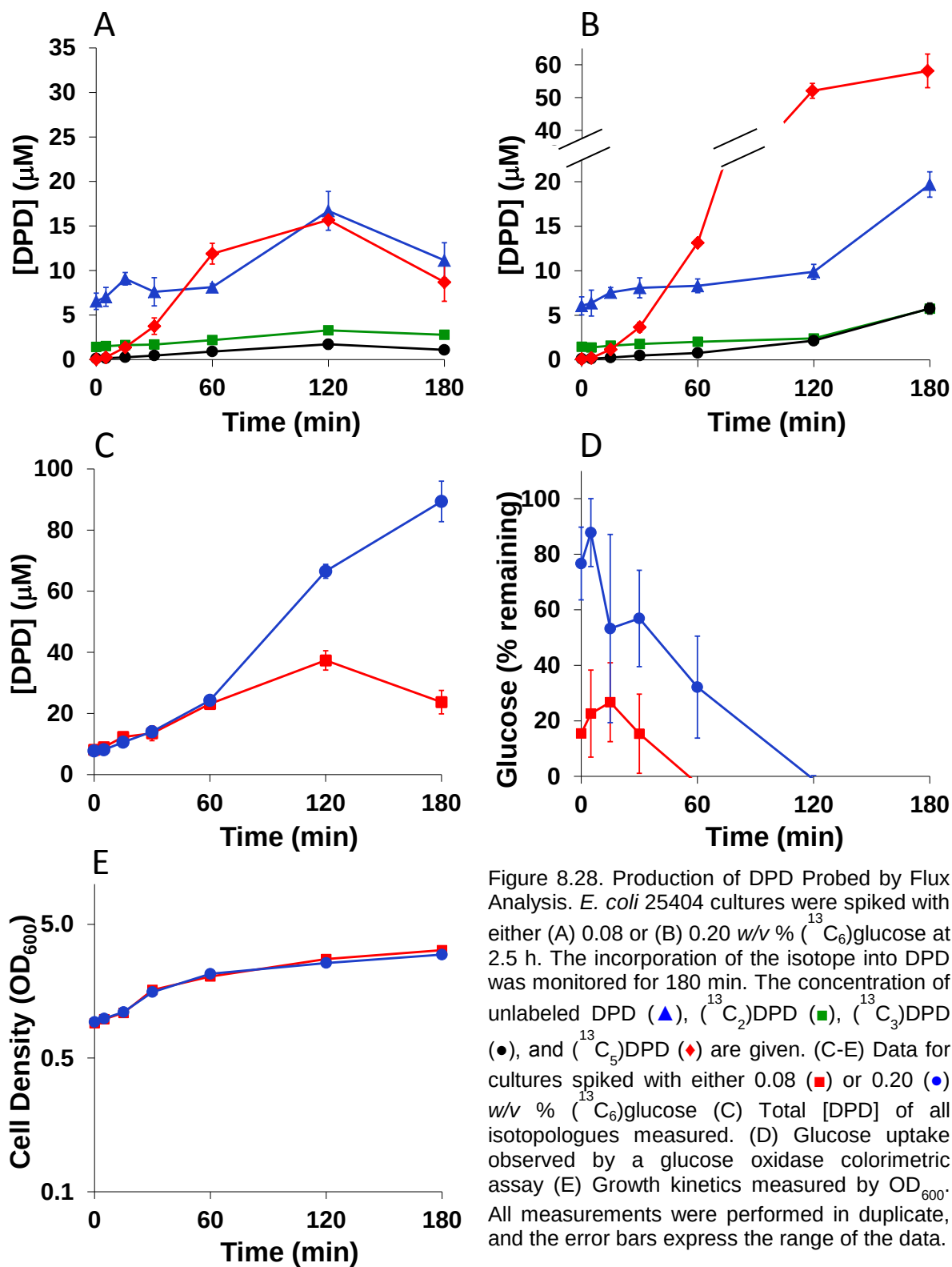


Figure 8.28. Production of DPD Probed by Flux Analysis. *E. coli* 25404 cultures were spiked with either (A) 0.08 or (B) 0.20 w/v % ($^{13}\text{C}_6$)glucose at 2.5 h. The incorporation of the isotope into DPD was monitored for 180 min. The concentration of unlabeled DPD (▲), ($^{13}\text{C}_2$)DPD (■), ($^{13}\text{C}_3$)DPD (●), and ($^{13}\text{C}_5$)DPD (◆) are given. (C-E) Data for cultures spiked with either 0.08 (■) or 0.20 (●) w/v % ($^{13}\text{C}_6$)glucose (C) Total [DPD] of all isotopologues measured. (D) Glucose uptake observed by a glucose oxidase colorimetric assay (E) Growth kinetics measured by OD₆₀₀. All measurements were performed in duplicate, and the error bars express the range of the data.

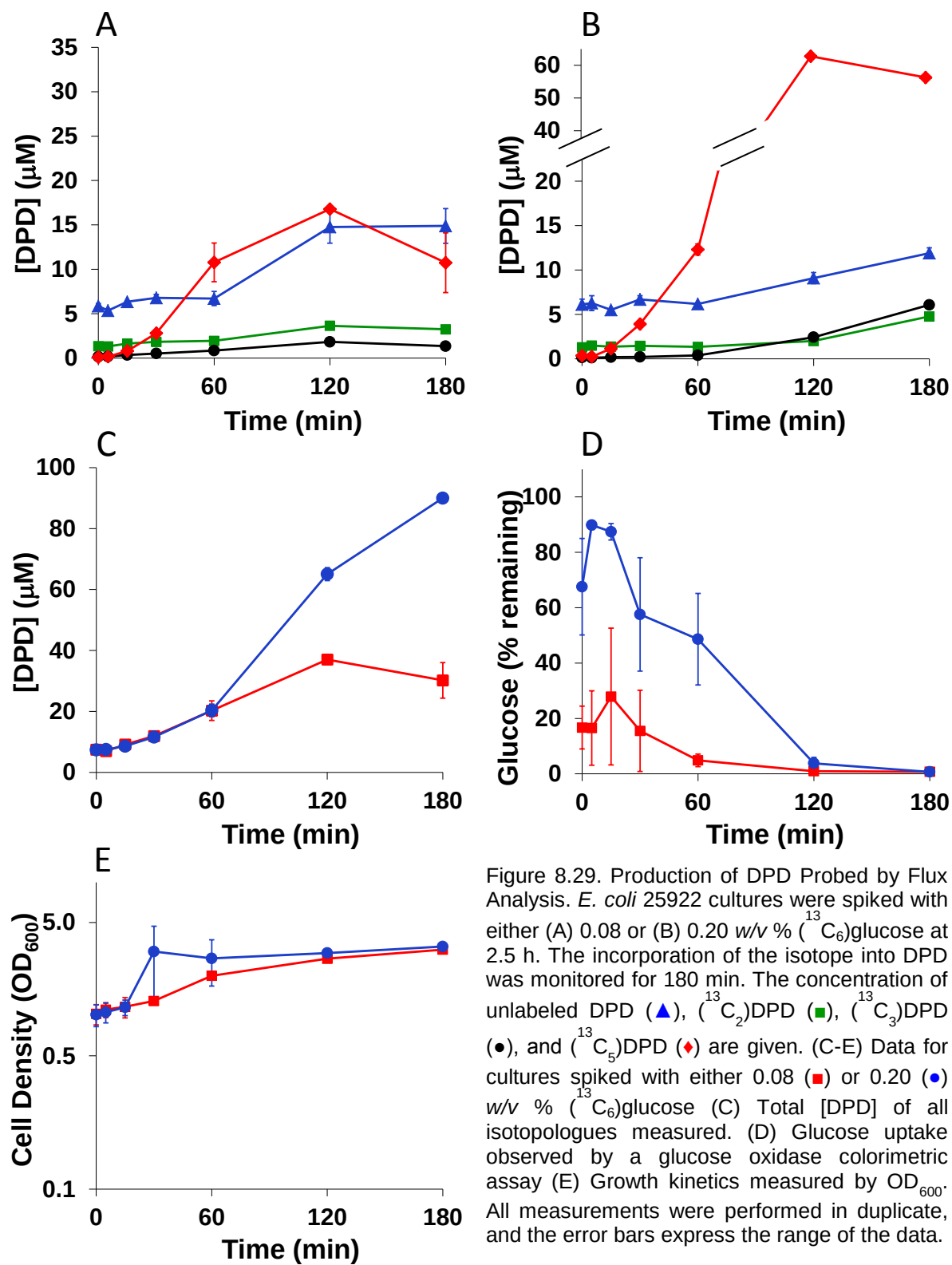


Figure 8.29. Production of DPD Probed by Flux Analysis. *E. coli* 25922 cultures were spiked with either (A) 0.08 or (B) 0.20 w/v % ($^{13}\text{C}_6$)glucose at 2.5 h. The incorporation of the isotope into DPD was monitored for 180 min. The concentration of unlabeled DPD (▲), ($^{13}\text{C}_2$)DPD (■), ($^{13}\text{C}_3$)DPD (●), and ($^{13}\text{C}_5$)DPD (◆) are given. (C-E) Data for cultures spiked with either 0.08 (■) or 0.20 (●) w/v % ($^{13}\text{C}_6$)glucose (C) Total [DPD] of all isotopologues measured. (D) Glucose uptake observed by a glucose oxidase colorimetric assay (E) Growth kinetics measured by OD₆₀₀. All measurements were performed in duplicate, and the error bars express the range of the data.

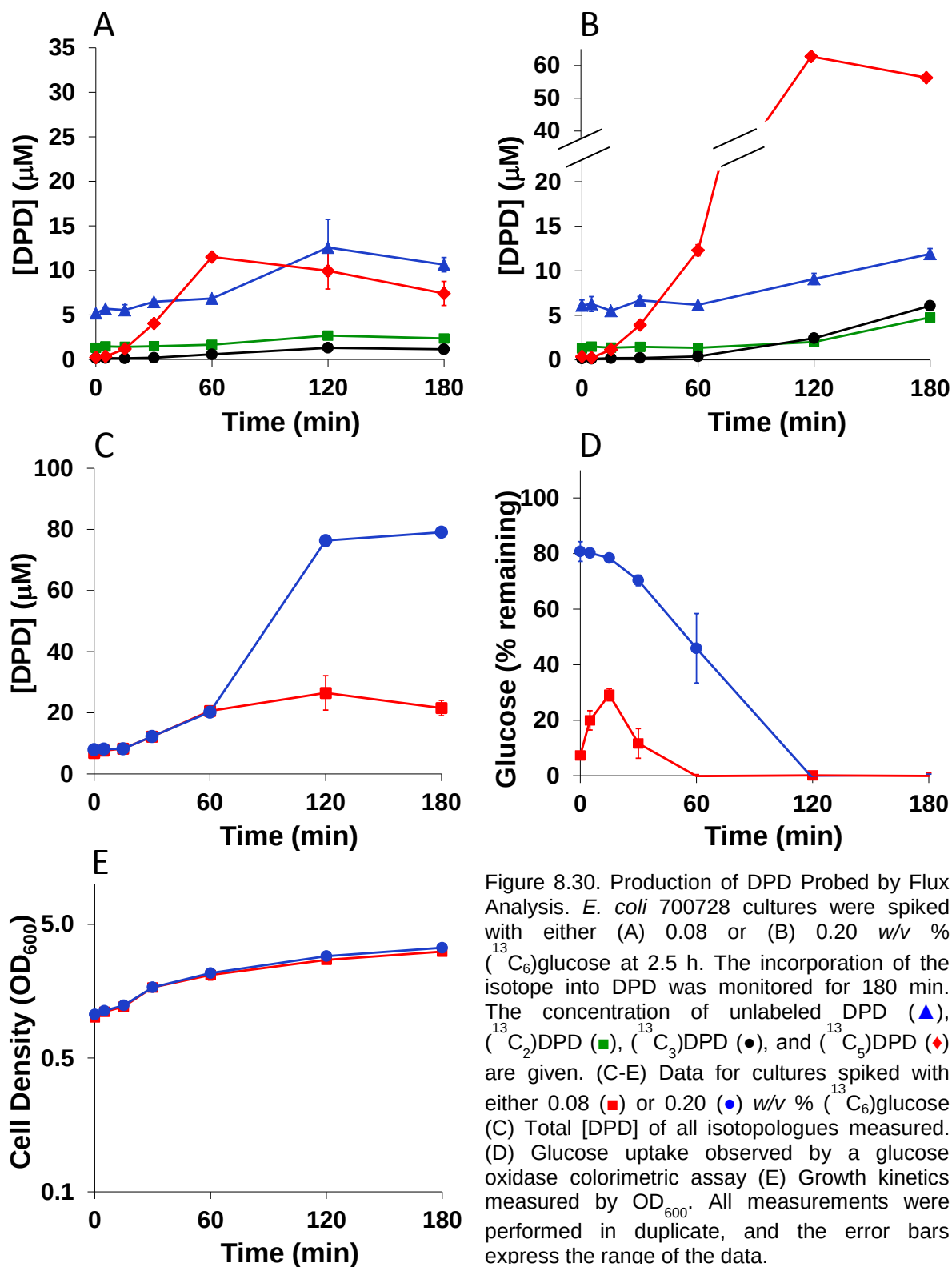


Figure 8.30. Production of DPD Probed by Flux Analysis. *E. coli* 700728 cultures were spiked with either (A) 0.08 or (B) 0.20 w/v % ($^{13}\text{C}_6$)glucose at 2.5 h. The incorporation of the isotope into DPD was monitored for 180 min. The concentration of unlabeled DPD ($\blacktriangle$), ($^{13}\text{C}_2$)DPD ($\blacksquare$), ($^{13}\text{C}_3$)DPD ($\bullet$), and ($^{13}\text{C}_5$)DPD ($\blacklozenge$) are given. (C-E) Data for cultures spiked with either 0.08 ($\blacksquare$) or 0.20 ($\bullet$) w/v % ($^{13}\text{C}_6$)glucose (C) Total [DPD] of all isotopologues measured. (D) Glucose uptake observed by a glucose oxidase colorimetric assay (E) Growth kinetics measured by OD_{600} . All measurements were performed in duplicate, and the error bars express the range of the data.

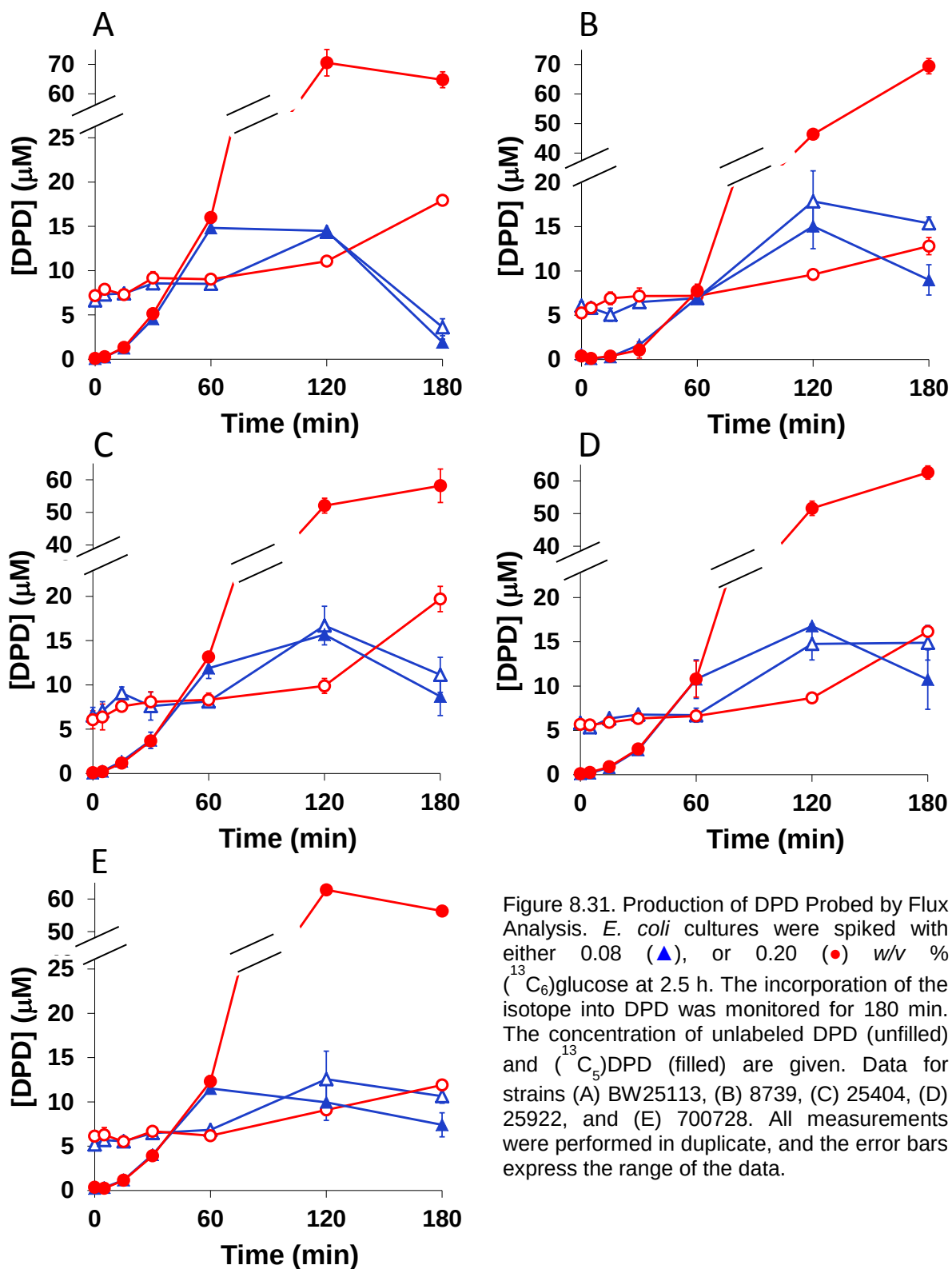


Figure 8.31. Production of DPD Probed by Flux Analysis. *E. coli* cultures were spiked with either 0.08 (▲), or 0.20 (●) w/v % (¹³C₆)glucose at 2.5 h. The incorporation of the isotope into DPD was monitored for 180 min. The concentration of unlabeled DPD (unfilled) and (¹³C₅)DPD (filled) are given. Data for strains (A) BW25113, (B) 8739, (C) 25404, (D) 25922, and (E) 700728. All measurements were performed in duplicate, and the error bars express the range of the data.

that excess DPD concentrations at higher concentrations of glucose are due to upregulated biosynthesis.

8.5 Extension to other species

Once we had validated our quantitative definition of quorum sensing in two model species, we then explored its predictive value in less characterized organisms. The following are incomplete data sets, however they demonstrate other examples of signaling behaviors. Most of this work was completed by Amanda May and MaryBeth Eisenhauer.

Edwardsiella tarda does not seem to regulate DPD production much like *E. coli* (Figure 8.32 panel A). *Yersinia enterocolitica* regulates the timing of signal production and only responds to added glucose by making slightly more DPD in BHI media. This species may use DPD to signal a timing event (panel B). *V. fischeri* makes at least 3 AHLs and DPD. The timing and concentrations are different for each signal, creating a cascade effect (panel C). The production of one signal regulates the production of later signals (data not shown), and at least the C6-homoserine lactone is another example of a timing signal. These data corroborates previously characterized enzyme regulation in *V. fischeri*, lending some support to the predictive value of our definition and regulation hypothesis. More work must be done however to truly characterize the predictive value of our definition.

8.6 Conclusions

We believe a quantitative approach can provide valuable insight into quorum sensing signal utilization. Our quantitative definition of quorum sensing produced results consistent with the literature. *V. harveyi* can regulate autoinducer-2 activity maintaining the reliability of the cell-density information it carries. On the other hand, *E. coli* production is modulated by nutritional status making the signal prone to environmental noise. We extended our approach to include stable isotope flux profiling and further found that regulation of signal production is accomplished through biosynthetic control in *V. harveyi*, whereas *E. coli* biosynthesis is upregulated under the conditions tested. *V. harveyi* does not respond to extracellular DPD sources, but the Lsr transport complex is induced early in *E. coli* at high DPD concentrations. We also found evidence of an

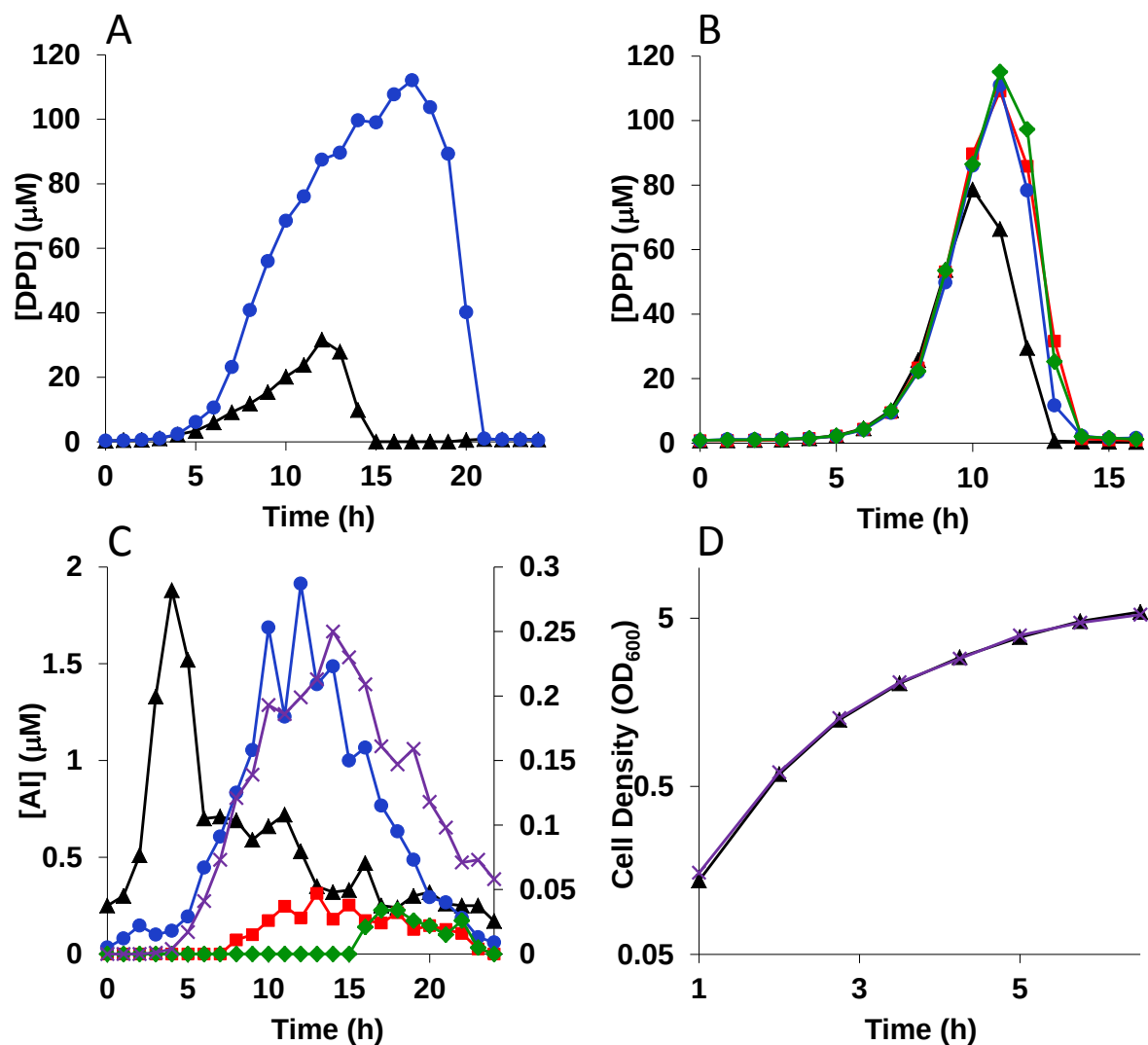


Figure 8.32. Autoinducer production in other bacterial species. (A) Measured DPD concentrations (μM) in *E. tarda* in 0.00 ($\blacktriangle$) or 0.14 ($\bullet$) % w/v glucose supplemented LB. (B) Measured DPD concentrations (μM) in *Y. enterocolitica* in 0.00 ($\blacktriangle$), 0.08 ($\blacksquare$), 0.14 ($\bullet$), or 0.20 ($\blacklozenge$) % w/v glucose supplemented BHI. (C) Measured DPD ($\blacktriangle$), C6- ($\blacksquare$), 3OC6- ($\bullet$), C7- ($\blacklozenge$), and C8- ($\times$) HSL concentrations (μM) in *V. fischeri* MJ-1 in LM. AHLs are on the secondary axis.

alternative import or degradation mechanism in Lsr transport complex mutants in the process of these experiments.

Similarly, we found that HAI-1 is produced at a constant rate, but not held at a constant ratio with cell density, leading us to believe it is actually a viability signal. Studies in other species of bacteria have demonstrated other possible types of signaling such as timing, but as of yet there is much work to be done characterizing these species before validating these results.

8.7 Methods

General Methods. Optical densities were measured on a BioPhotometer (Eppendorf), and all absorbance measurements for the glucose oxidase assay were performed on a μ QUANT Universal Microplate Spectrophotometer (Bio-Tek Instruments, Inc.). The liquid chromatography-tandem mass spectrometer (LC-MS/MS) used for these studies consisted of either a Surveyor Autosampler Plus (Thermo Electron Corporation), a Surveyor MS Pump Plus (Thermo Electron Corporation), or an Agilent 1100 Series HPLC and a TSQ Quantum Discovery Max triple quadrupole MS (Thermo Electron Corporation). All MS spectra were collected and analyzed using the Xcalibur MS software package (Thermo Electron Corporation). The stationary phase of the column was aminopropyl functionalized particles (5 μ m pore size, 100 Å particle size) packed into a 250 $\times$ 2 mm column (Phenomenex Luna NH2) for the DPD analysis and a Gemini-NX C18 (5 μ m pore size, 110 Å particle size) packed into a 150 $\times$ 2 mm column (Phenomenex) for the HAI-1 Analysis. (S)-4,5-Dihydroxy-2,3-pentanedione (DPD)¹⁶⁹, (1-¹³C₁)-(S)-4,5-Dihydroxy-2,3-pentanedione ((1-¹³C)DPD)¹⁶⁹ and the derivatizing agent, dimethyl 2,2'-(4,5-diamino-1,2-phenylene)bis(oxy)diacetate • xHCl (M1CQ)²¹⁰ were prepared according to reported methods. The following chemicals were commercially available as an ACS grade compound and used without further purification unless otherwise noted: water (HPLC, VWR International), sodium dihydrogen phosphate dihydrate (99+%, Fluka Chemical), glycerol (J.T. Baker), and sulfuric acid (Mallinckrodt). All other reagents listed below were ordered from Fisher Scientific: Acetonitrile (HPLC), ethyl acetate, sodium chloride, cyclohexanone, tris(hydroxymethyl)aminomethane (Tris) (99+ %), kanamycin monosulfate (biotech reagent), tryptone, yeast extract, glucose, o-dianisidine, peroxidase, and glucose oxidase type X.

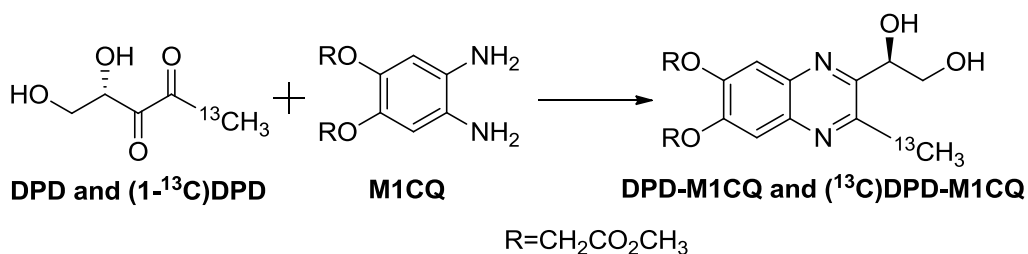
Bacterial Strains and Growth Conditions. *Escherichia coli* BW25113, JW2662-1 (*luxS*⁻), JW1506-2 (*lsrA*⁻), JW1509-1(*lsrB*⁻), JW1507-2 (*lsrC*⁻), and JW1508-1 (*lsrD*⁻) were established as part of the Keio Collection²¹² and purchased from the Coli Genetic Stock Center (Yale University). *Escherichia coli* ATCC 8739, ATCC 25404, ATCC 25922, and ATCC 700728 were purchased as lyfocults from VWR. Cultures of *E. coli* were grown aerobically in Luria broth (LB)¹⁸⁹ at 37°C with shaking (~100 rpm). *Vibrio harveyi* BB120 (wild type)¹⁵⁴ and MM30 (*luxS*⁻)¹⁵⁵ were a gift from Bonnie L. Bassler (Princeton University and the Howard Hughes Medical Institute). Cultures of *V. harveyi* were grown aerobically in Luria marine medium (LM)¹⁵³ at 30°C with shaking (~100 rpm).

Chromatographic Details for DPD detection. High performance liquid chromatography (HPLC) was performed utilizing a quaternary pump to generate a gradient for the elution of compounds from the stationary phase. For all samples, 10 µL was injected onto the column via an autosampler cooled to 4 °C. A flow rate of 150 µL / min was used, and the eluent was introduced directly into the MS for ion detection. The mobile phases were HPLC grade water (solvent A) and HPLC grade acetonitrile (solvent B), and these were used to construct the following 18 min gradient elution profile: (t) 0 min, 15% solvent A, 85% solvent B; t) 2 min, 15% solvent A, 85% solvent B; t) 4 min, 95% solvent A, 5% solvent B; t) 14, 95% solvent A, 5% solvent B; t) 16, 15% solvent A, 85% solvent B; t) 18, 15% solvent A, 85% solvent B). All separations were performed with the column at ambient temperature.

Chromatographic Details for HAI-1 detection. High performance liquid chromatography (HPLC) was performed utilizing a quaternary pump to generate a gradient for the elution of compounds from the stationary phase. For all samples, 10 µL was injected onto the column via an autosampler. A flow rate of 150 µL / min was used, and the eluent was introduced directly into the MS for ion detection. The mobile phases were 0.1% Acetic Acid (solvent A) and HPLC grade acetonitrile (solvent B), and these were used to construct the following 13 min gradient elution profile: (t) 0 min, 95% solvent A, 5% solvent B; t) 1 min, 95% solvent A, 15% solvent B; t) 3 min, 50% solvent A, 50% solvent B; t) 8 min, 50% solvent A, 50% solvent B; t) 10 min, 5% solvent A, 95%

solvent B; t) 12 min, 5% solvent A, 95% solvent; B t) 0 min, 95% solvent A, 5% solvent B). All separations were performed with the column at 25°C.

General Mass Spectrometric Detection Parameters. Samples were introduced into the electrospray ionization (ESI) chamber of the triple quadrupole MS through a 0.1 mm internal diameter fused silica capillary after delivery by HPLC as described above. The spray voltage for the ESI source was set to 4500 V, and detection occurred in positive ion mode. Nitrogen was used as the sheath gas (40 psi), and the inlet capillary temperature was 290 °C. The argon used as the collision gas was set at 1.5 mTorr. Samples were analyzed using selected reaction monitoring (SRM), and the scan time for each SRM was 0.05 s with a scan width of 1 *m/z*.



Measurement of DPD Concentration ([DPD]) by Derivatization with a Substituted 1,2-Diaminobenzene. Samples were prepared as previously reported²¹⁰ with only the minor addition of an extraction step. At each timepoint, a 300 µL aliquot of the culture was sampled and then added to a 1.5 mL microcentrifuge tube containing a solution of the (¹³C₁)-(S)-4,5-Dihydroxy-2,3-pentanedione internal standard ((¹³C)DPD) (341 µM in 10 µL H₂O) to give a 310 µL volume solution (solution A) containing 11 µM internal standard. The contents were thoroughly mixed by vortexing and then centrifuged (13,200 rpm, 16,100 rcf, 1 min) to remove cells and other particulates. A portion of the resulting supernatant (260 µL) was then transferred to a separate 1.5 mL microcentrifuge tube containing a solution of derivatizing agent, dimethyl 2,2'-(4,5-diamino-1,2-phenylene)bis(oxy)diacetate • xHCl (M1CQ) (14 mM in 26 µL H₂O) to give a 286 µL volume solution (solution B) containing 1.3 mM M1CQ. The two liquids were thoroughly mixed by vortexing, and reaction was allowed to proceed for 45 min at ambient temperature. At this point, the reaction mixture (solution B) was extracted twice with 130 µL ethyl acetate, and the organic layers were combined in a 300 µL screwcap

autosampler vial. Samples were stored at 4 °C until MS analysis. Note: Extraction with ethyl acetate removes excess salts and derivatizing reagent from the reaction samples without hindering quantitation due to the use of an internal standard. This purification step minimizes the amount of contaminants that are introduced into the MS.

Measurement of HAI-1 Concentration ([HAI-1]). At each timepoint, a 300 µL aliquot of the culture was sampled and then added to a 1.5 mL microcentrifuge tube containing a solution of the *N*-(3-hydroxybutanoyl)-L-homoserine lactone internal standard ((D₂)HAI-1) (526 µM in 11.4 µL H₂O) to give a 310 µL volume solution containing 19.25 µM internal standard. The contents were thoroughly mixed by vortexing and then centrifuged (13,200 rpm, 16,100 rcf, 1 min) to remove cells and other particulates. A portion of the resulting supernatant (260 µL) was then transferred to a separate 1.5 mL microcentrifuge. The supernatants were then extracted twice with 130 µL ethyl acetate, and the organic layers were combined in a 300 µL screwcap autosampler vial. Samples were then used immediately for MS analysis. Note: Extraction with ethyl acetate removes excess salts from the reaction samples without hindering quantitation due to the use of an internal standard. This purification step minimizes the amount of contaminants that are introduced into the MS. Acyl homoserine lactones have been readily detected via LC/MS methods,²¹³⁻²¹⁹ and our method had a limit-of-detection (LOD) for (D₂)HAI-1 of 5.3 nM and a linear range from 5.3 nM to 66 µM.

Data Handling for [DPD]. Data from SRMs 381-202 and 382-203 (parent-product ion) with a collision energy of 43 eV were selective for the quinoxalines DPD-M1CQ and (¹³C)DPD-M1CQ respectively and were used to calculate [DPD]. Chromatographic peaks were manually integrated over the same time interval within a sample. Integrations were then corrected by one of two methods depending on the ratio of (¹²C)DPD-M1CQ signal to (¹³C)DPD-M1CQ signal. Correction Type 1 is a correction for (¹²C)DPD impurities in the (1-¹³C)DPD internal standard and was applied when the ratio of (¹²C)DPD-M1CQ to (¹³C)DPD-M1CQ was less than 0.2

$$\begin{aligned} \text{Corrected Signal } (^{12}\text{C})\text{DPD-M1CQ} \\ = \text{Observed Signal DPD-M1CQ} - 0.008 \\ \times \text{Observed Signal } (^{13}\text{C})\text{DPD-M1CQ} \end{aligned}$$

Correction Type 2 is a correction for naturally occurring ($^{13}\text{C}_1$)DPD or other [M+1] DPD molecules and was applied when the ratio of DPD-M1CQ to ($^{13}\text{C}_1$)DPD-M1CQ was greater than 0.3

$$\begin{aligned} \text{Corrected Signal } (^{13}\text{C}_1)\text{DPD-M1CQ} \\ = \text{Observed Signal } (^{13}\text{C})\text{DPD-M1CQ} - 0.144 \\ \times \text{Observed Signal } (^{12}\text{C})\text{DPD-M1CQ} \end{aligned}$$

After correction, the following formula was applied to calculate the [DPD] of the culture.

$$\begin{aligned} \frac{\text{Signal DPD-M1CQ}}{\text{Signal } (^{13}\text{C})\text{DPD-M1CQ}} \times [(1-^{13}\text{C})\text{DPD in solution A}] \times \frac{\text{volume solution A}}{\text{volume culture sampled}} \\ = [\text{DPD}] \end{aligned}$$

For experiments measuring isotopologues, the additional SRMs 383-203, 384-205, 386-206 were monitored for quantitation of ($^{13}\text{C}_2$)DPD-M1CQ, ($^{13}\text{C}_3$)DPD-M1CQ, and ($^{13}\text{C}_5$)DPD-M1CQ respectively. Corrected ion counts for ($^{13}\text{C}_1$)DPD-M1CQ were still used due to the presence of DPD in solution. Isotopologue concentrations were then calculated using the following formula.

$$\begin{aligned} \frac{\text{Signal Isotopologue}}{\text{Signal } (^{13}\text{C})\text{DPD-M1CQ}} \times [(1-^{13}\text{C})\text{DPD in solution A}] \times \frac{\text{volume solution A}}{\text{volume culture sampled}} \\ = [\text{DPD}] \end{aligned}$$

Data Handling for [HAI-1]. Data from SRMs 188-102 and 190-104 (parent-product ion) with a collision energy of 11 eV were selective for the HAI-1 and (D_2)HAI-1 respectively and were used to calculate [HAI-1]. Chromatographic peaks were manually integrated over the same time interval within a sample.

The following formula was applied to calculate the [HAI-1] of the culture.

$$\frac{\text{Signal HAI-1}}{\text{Signal } (D_2)\text{HAI-1}} \times [(D_2)\text{HAI-1}] = [\text{HAI-1}]$$

Establishment of an External Calibration Curve for Colorimetric Glucose Oxidase

Assay. A set of wells containing 110 μL solutions of known glucose concentrations ([glucose]) were established on each assay plate so that an external calibration curve could be constructed to allow quantitation. This set of calibration solutions contained 0.3960, 0.1980, 0.0990, 0.0495, 0.0248, 0.0124, 0.0062, and 0 w/v % glucose and were generated by diluting 2.2 μL of a 40 w/v % glucose stock solution into 220 μL LB followed by two-fold serial dilutions. Aliquots (12 μL) of the calibration curve were transferred to different wells and diluted with 90 μL LB such that each sample plate had two calibration curves at two different sets of concentrations (undiluted and 12:102 dilution). This dilution step was necessary to fit the higher glucose concentrations into the linear range of the assay. The calibration standards for each plate were used to generate a calibration equation by least-squares regression of the linear portion of the data (0 to 0.0248 % for the undiluted curve and 0 to 0.0990 % for the diluted curve) of the form:

$$A_{500} = m \times [\text{glucose}] + b$$

This equation was then used to determine the glucose concentrations from the absorbance measured for each sample. The undiluted calibration curve and samples were used to calculate [glucose] for all experiments performed without the addition of glucose. When either 0.08, 0.14, or 0.20 w/v % glucose was added to the culture, the diluted calibration curve was used as the samples also required dilution to bring the values into linear range. The assay was not found to be quantitative, however it was qualitatively useful. [Glucose] was therefore normalized to the highest concentration (0.2 % added) and reported as % added glucose remaining.

Measurement of Glucose Concentrations by Colorimetric Glucose Oxidase Assay.

For each time point, a 150 μL aliquot of the culture was added to a 1.5 mL microcentrifuge tube and centrifuged (13,200 rpm, 16,100 rcf, 1 min) to remove cells and other particulates. At this time, 130 μL of the supernatant was transferred to a new 1.5 mL microcentrifuge tube, and the samples were immediately frozen and stored at -80°C

until the assay was performed (no longer than 48 h after collection). After all samples were collected, a 112 μL aliquot of each was transferred to a clear, flat-bottomed, 96-well plate. Aliquots (12 μL) of the cell supernatants were then transferred to different well and diluted with 90 μL LB such that each sample was measured at two different concentrations (undiluted and 12:102 dilution). A 200 μL aliquot of an assay reagent²²⁰ containing 0.30 M Tris, 0.36 M $\text{NaH}_2\text{PO}_4 \bullet 2\text{H}_2\text{O}$, 5.4 M glycerol, 1.0 mM *o*-dianisidine, 3 kU/ L peroxidase and 1 kU/L glucose oxidase type X in water was added to each well and the plate was incubated for 15 h at 37 °C. At this time, the absorbance at 500 nm was measured, and the [glucose] was calculated according to the method described above.

Measurement of Cell Density by Optical Density. For early time points ($t = 0 - 2.75$ h *E. coli* or $t = 0 - 3.5$ h *V. harveyi*), cell density was determined by measuring the OD_{600} of 700 μL aliquots of each culture, a 300 μL portion of this aliquot was then used to measure [DPD], and a 150 μL portion of this aliquot was used to determine [glucose]. For later time points, the optical density was above the linear range of the spectrometer, therefore a 300 μL aliquot of each culture was mixed with 900 μL of fresh media, mixed and cell density was determined by measuring the OD_{600} of the diluted samples. For points that required dilution, separate aliquots of 300 μL and 150 μL were taken directly from the culture for the determination of [DPD] and [glucose] respectively and used immediately. All [DPD] and [glucose] measurements were performed as described above.

Measurement of Culture Viability. A 5 mL culture of *V. harveyi* was grown overnight in LM. The overnight culture was then used to make 2 v/v % inocula in each of two 125 mL glass Erlenmeyer flasks by diluting 500 μL of the culture into 25 mL LM supplemented with either 0.00 or 0.14 w/v % glucose. After 6.5 h of growth, aliquots (10 μL) were diluted 1:100 into LM and 8 ten-fold serial dilutions were made (10 μL into 100 μL LM). Aliquots (10 μL) of each dilution were then spread on LM plates in duplicate and incubated at 30°C overnight. The number of colonies was then counted and converted to colony forming units (CFU) per mL using the following equation.

$$\text{Number of colonies} \times \text{dilution factor} \div 10 \mu\text{L} \times 1000 \mu\text{L} / \text{mL}$$

Monitoring Extracellular AI-2 Under Varying Nutrient Conditions. For each experiment, a 5 mL culture of the strain of interest was grown overnight in appropriate media as described above. The overnight cultures were then used to make 2 v/v % inocula in each of four 125 mL glass Erlenmeyer flasks by diluting 500 μ L of the culture into 25 mL LB (*E. coli*) or LM (*V. harveyi*) supplemented with either 0.00, 0.08, 0.14, or 0.20 w/v % glucose. Cultures were sampled for [DPD] and [glucose] at the time intervals indicated and analyzed as described above. For glycerol experiments 0.00, 0.07, or 0.17 w/v % glycerol was added to each Erlenmeyer flask, and sampled for [DPD] only.

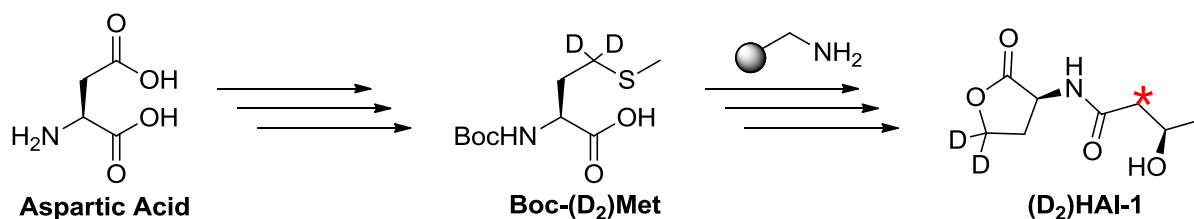
Monitoring Extracellular *V. harveyi* Autoinducer-1 Under Varying Nutrient Conditions. For each experiment, a 5 mL culture of the strain of interest was grown overnight in appropriate media as described above. The overnight cultures were then used to make 2 v/v % inocula in each of four 125 mL glass Erlenmeyer flasks by diluting 500 μ L of the culture into 25 mL LM supplemented with either 0.00, 0.08, 0.14, or 0.20 w/v % glucose. Cultures were sampled for [HA1-1] and [glucose] at the time intervals indicated and analyzed as described above.

Flux Profiling of Nutrient Effects on DPD Biosynthesis. Two overnight cultures of the strain to be tested were grown overnight in either LB or LM for *E. coli* or *V. harveyi* strains, respectively. A 500 μ L aliquot of the overnight cultures were then used to make 2 v/v % inocula in each of four 125 mL glass Erlenmeyer flasks containing 25 mL LB (*E. coli*) or LM (*V. harveyi*). After 2.5 h (*E. coli*) or 3 h (*V. harveyi*) of growth, 0.04 or 0.14 w/v % ($^{13}\text{C}_6$)glucose (25 and 88 μ L of 40 w/v% in H_2O) was added to each culture. Cultures were sampled at the time intervals indicated and analyzed as described above.

Flux Profiling of Exogenous DPD Effects on DPD Biosynthesis. Two overnight cultures of *V. harveyi* to be tested were grown overnight in LM. Aliquots (500 μ L) of the overnight cultures were then used to make 2 v/v % inocula in each of four 125 mL glass Erlenmeyer flasks containing LM. After 3 h of growth, 0.14% glucose (40 w/v% in 88 μ L H_2O) was added to each culture, as well as either synthetic (^{12}C)DPD to make 28 μ M solutions of exogenous DPD (133 μ L of 4.7 mM DPD in H_2O) or a control solution of acidic cyclohexanone (133 μ L of 4.7 mM cyclohexanone pH=1 in H_2O) to account for

other constituents of the synthetic DPD solution. Cultures were sampled at the time intervals indicated and analyzed as described above.

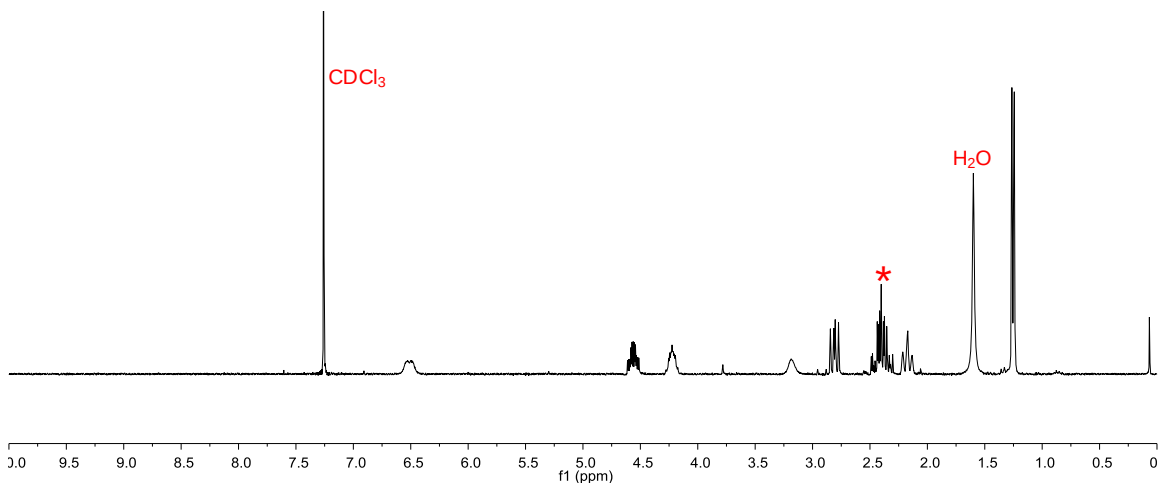
Generation of 3-Dimensional Plots Displayed in Figure 1. A Microsoft Excel 2007 spreadsheet was created containing columns for time (h), initial [glucose] added (w/v %), [AI]/OD₆₀₀ (μM/OD₆₀₀) replicate 1, [AI]/OD₆₀₀ (μM/OD₆₀₀) replicate 2, and the calculated average of [AI]/OD₆₀₀ (μM/OD₆₀₀). These data were then imported into a MATLAB 7.9 workspace as vectors. The plot3 function was used to plot the average [AI]/OD₆₀₀ data. The meshgrid, griddata, and mesh functions were then used to create the mesh surface with time on the x axis, initial [glucose] added on the y axis, and average [AI]/OD₆₀₀ on the z axis. The higher of the two values measured for the [AI]/OD₆₀₀ from the replicate experiments was graphed to indicate the upper range of the error. These points are displayed as error bars generated with the plot3 function. Because of the opaqueness of the surface, the lower error bars were not graphed. However, since the [AI]/OD₆₀₀ data is averaged from two points and the error indicates the range, the magnitude of the upper and lower error bars is equal.



Synthesis of (D₂)HAI-1 internal standard. Aspartic acid was converted to *tert*-butoxycarbonyl protected dueterated methionine (Boc-(D₂)Met) through a modified synthesis of several known reactions.^{221,222} This was then coupled to an aminomethyl polystyrene resin using modified versions of known coupling conditions.^{223,224} Finally, (D₂)HAI-1 was produced by the method of Kim and Ko which relies on several standard solid phase reactions. This procedure yielded (D₂)HAI-1 in >97 % purity after silica column as judging by ¹HNMR. A detailed synthesis of this molecule will be communicated in a forthcoming publication. The ¹HNMR of the final molecule matched the previously published spectrum²²⁵ with the exception of the protons replaced by deuteriums were not observed. ¹HNMR (300 MHz, D₂O) δ 4.52 (dd, J = 22.1, 11.5 Hz,

1H), 4.14 – 3.96 (m, 1H), 2.52 – 2.40 (m, 1H), 2.30 (d, J = 7.2 Hz, 2H), 2.25 – 2.09 (m, 1H), 1.07 (d, J = 6.3 Hz, 3H).

To observe the exchangeable protons and show purity, the ^1H NMR in CDCl_3 was also taken. The chemical shifts and splitting patterns are similar those observed in D_2O with the exception of the protons on carbon * which appear as a doublet in D_2O and a multiplet in CDCl_3 . Presumably, this is due to hydrogen bonding between the hydroxyl group and the ketone in an aprotic solvent which forces a more rigid conformation of the molecule rendering the protons on carbon * diastereotopic. ^1H NMR(300 MHz, CDCl_3) δ 6.53 (s, 1H), 4.67 – 4.42 (m, 1H), 4.33 -4.12 (s, 1H), 3.19 (bs, 1H), 2.81 (dd, J = 12.5, 8.7 Hz, 1H), 2.53 – 2.26 (m, 2H), 2.17 (t, J = 12.3 Hz, 1H), 1.26 (dd, J = 6.3, 1.0 Hz, 3H). HRMS-DART (m/z): $[\text{M}+\text{H}]^+$ calculated for $\text{C}_8\text{H}_{12}\text{D}_2\text{NO}_4$ 190.10429; found, 190.10352.



CHAPTER IX THE FUNCTION OF AI-2

9.1 Background

Considering AI-2 has been implicated in so many important processes, it is interesting that the function of this molecule is still much debated (see Chapter 6). Popular belief says AI-2 is an interspecies quorum sensing signal, a bacterial Esperanto.²²⁶ Indeed, there is much evidence to suggest this is a signal in particular species; however, our studies of *E. coli* indicated that it is not likely to use AI-2 as a signal, since it does not regulate DPD biosynthesis.

Another hypothesis points to the metabolic necessity of maintaining an intact activated methyl cycle and posits that elimination of LuxS function would cause a global metabolic disruption, and third hypothesis states that *E. coli* does not use AI-2 signaling but imports the signal as a defense mechanism. Herein, each of these hypotheses is tested in an effort to assign a function to AI-2 for species that do not use it as a quorum sensing signal.

9.2 Validating the Biological Relevance of Metabolomics Experiments

We first focused our attention on the metabolic hypothesis, and used our metabolomics methods to test whether a *luxS*⁻ mutation could cause a metabolic disruption large enough to explain the conservation of LuxS. Metabolomic studies are best done in minimal media to avoid carry over contamination of the extracts with media components. We therefore first performed a few control studies to support the method design. First BW25113 DPD production was monitored in minimal media using the methods reported in Chapter 8 (Figure 9.1). The concentration reached 17 μ M in 8 h of growth. This concentration is comparable to the peak concentration produced by BW25113 in LB (19 μ M). The slowed production of DPD (compared to 3.5 h) is explained by the slower growth in minimal media. When cell density v. [DPD] is plotted, the picture changes. BW25113 actually produces more DPD per cell in minimal media than when grown in 0.2% supplemented LB, probably due to the 0.4% w/v glucose it contains. We were

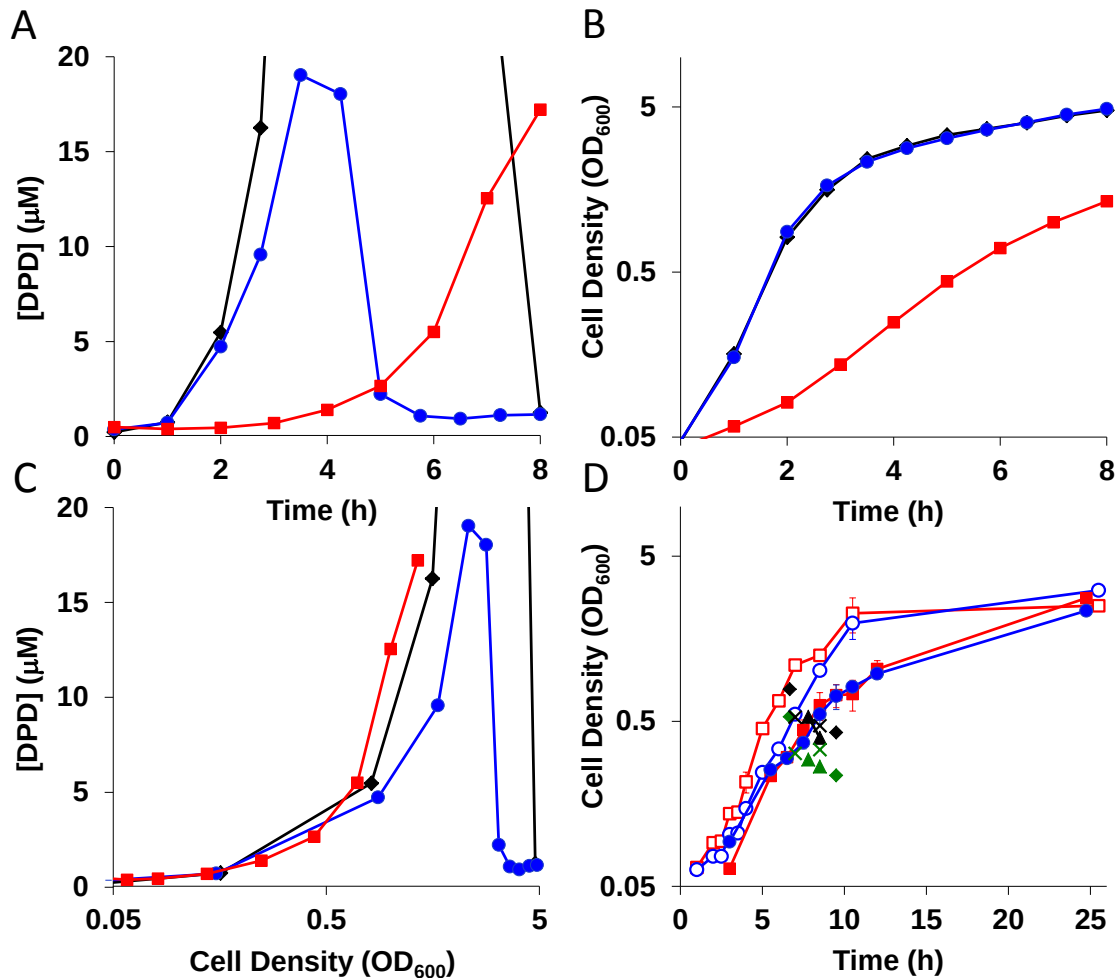


Figure 9.1. *E. coli* growth and DPD production in minimal media. A) DPD production and B) growth kinetics of wild type *E. coli* in minimal media (■), LB (●), or LB + 0.2% w/v glucose (◆). If DPD production is plotted against cell growth (C), minimal makes DPD at a faster rate. D) Growth of wild type (■) and luxS (●) strains in minimal media (open) or on minimal agarose supported filters (filled). Actual times and optical densities for wild type (black) and luxS (green) cultures at just before sampling for carbon flux (♦), nitrogen flux (▲), and pool size (x) are also plotted.

therefore satisfied that high density phenotypes could be triggered in a minimal media environment.

The next control was meant to address another technical issue. Metabolism changes in seconds, so unattached cells are typically grown on a filter to facilitate speedy switching between nutrient sources and quenching of metabolism. A side-by-side comparison of liquid and filter growth was run by inoculating an LB + kanamycin maintained *E. coli luxS⁻* strain JW2662-1 and its LB maintained parent strain BW25113 1:50 into minimal media and acclimatized for 36 h with an inoculation into fresh media at 24 h. After 36 h, the bacteria were inoculated into each of two flasks containing fresh media, one for liquid culture measurements and one for filter culture measurements for a total of four flasks. Cell density was regularly monitored by optical density at 600 nm for liquid cultures. When cultures reached ~ 0.1 OD₆₀₀ (~ 3 h), filter cultures were established by adding 5 mL of culture to each filter and then transferring the filter face side up to a minimal agarose plate. Growth on these filters was monitored by regularly transferring a filter to 5 mL of water and flushing the bacteria from the filter by pipetting over the surface. The cell density of this solution was then measured by optical density at 600 nm (Figure 9.1 panel D). Note this experiment could have been better designed if an isotonic solution such as PBS or minimal media were used instead of water, as a change in osmolarity will affect the size and therefore light scattering behavior of the bacteria. We observed a slower growth rate for filter cultures when compared to the growth rate of liquid cultures. These findings disagree with previously published data from the Rabinowitz laboratory;⁵⁴ however, the osmolarity of the test solution may explain the observed difference. No major differences in growth rate were observed between WT and *luxS⁻* strains in either growth environment. However optical densities were frequently lower in *luxS⁻* strains compared to WT in later metabolomics experiments.

9.3 Metabolomics Experiments

Metabolomics were completed on six separate days following protocols adapted from the Rabinowitz laboratory.^{25,52} Carbon flux experiments were carried out in duplicate on two of these days, nitrogen flux in duplicate on another two days, and extra pool size experiments (or time = 0) in quadruplicate on the last two days for a total of 16 possible pool size observations. Experiments were completed by growing *E. coli* strain BW25113

or $\Delta luxS$ strain JW2662-1 on a minimal agarose media for 4-6 h after filter cultures were established. At this time, two filters for each strain was transferred into extraction solvent face down and kept at -20°C for 20 min for the time zero measurement. The other filters were then switched in a slightly staggered manner to minimal agarose plates containing either $(^{13}\text{C}_6)\text{glucose}$ or $^{15}\text{NH}_4\text{Cl}$. These filters were then quenched and extracted at regular time points (2, 5, 15, 30 and 60 min for ^{13}C or 2, 5, 10, 20, and 40 min for ^{15}N). Similar experiments were conducted with less rigor in the early stages of the project with different carbon sources, as well as with addition of methionine, DPD and epinephrine.

Xcalibur (Thermo Electron, Waltham MA) was used to pick, integrate, and match chromatographic peaks to a library of standards. The peaks were then manually checked for false positives, false negatives and poor quantitation due to quirks in the algorithm. The data were then normalized by optical density at inoculation, and internal standard signal by assuming a proportional relationship between each of these values and the integrated signal. Plane polarized light microscopy was performed to compare average cell size between the two strains (Figure 9.2). A difference in size between strains would affect the observed optical density. No statistically significant differences were discerned. Of the 94 metabolites measured there were 20 metabolites that had statistically changes by F-test (Figure 9.3 and Table 9.1). Of these only 8 had fold changes of 1.5 fold or more and these were the only metabolites considered to change.

Integrations observed in flux experiments were used to calculate the fraction of the

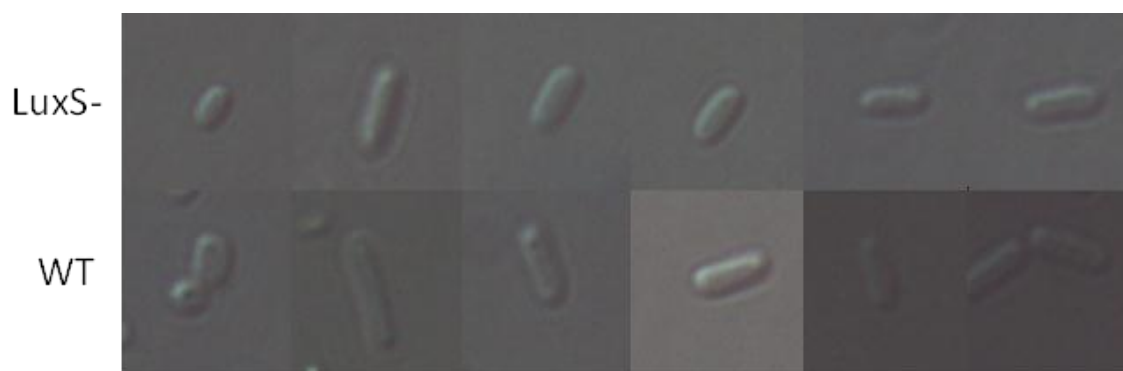


Figure 9.2. *E. coli* size and shape as observed via plane polarized light microscopy. Pictures are representative of the smallest, largest, and several mid-size bacteria.

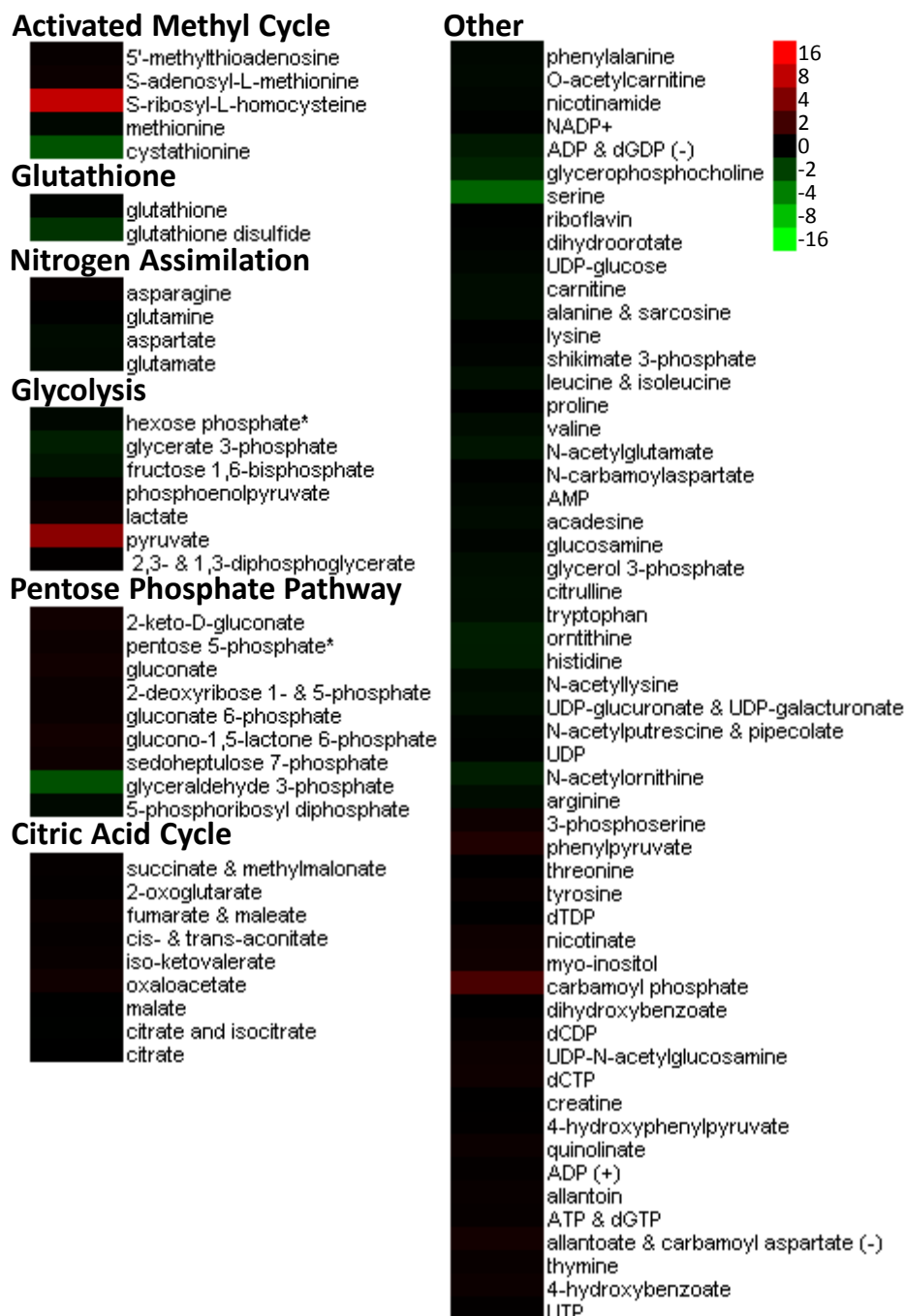


Figure 9.3. Heat map displaying the fold change in concentration when levels in the luxS mutant are compared to wild type concentrations. Red indicates the metabolite has accumulated in the mutant and green indicates a decrease.

Table 9.1 Metabolites Measured

Metabolite	Fold change in LuxS Mutant Relative to WT	p value	WT Times Measured	LuxS⁻ Times Measured
S-ribosyl-L-homocysteine (-)	37.73	1.32 x10 ⁻¹⁰	10	10
serine	-2.95	3.60 x10 ⁻⁷	10	12
S-ribosyl-L-homocysteine (+)	8.37	5.61 x10 ⁻⁷	12	16
pyruvate	4.55	4.09 x10 ⁻⁶	11	12
carbamoyl phosphate	2.21	3.19 x10 ⁻⁵	12	9
histidine	-1.37	4.79 x10 ⁻⁵	16	16
glycerate 3-phosphate	-1.40	6.74 x10 ⁻⁴	12	12
creatine	1.02	8.08 x10 ⁻⁴	11	9
glutathione disulfide	-1.76	8.77 x10 ⁻⁴	16	16
glyceraldehyde 3-phosphate	-2.41	8.86 x10 ⁻⁴	8	8
cystathionine	-2.49	3.23 x10 ⁻³	7	6
ornithine	-1.38	4.36 x10 ⁻³	16	16
glycerophosphocholine	-1.48	0.01	12	12
tryptophan	-1.17	0.01	16	16
ADP & dGDP (-)	-1.33	0.03	12	12
carnitine	-1.15	0.04	8	7
N-acetyllysine	-1.13	0.04	14	14
phenylalanine	-1.08	0.04	16	16
methionine	-1.09	0.04	16	14
glutamate	-1.11	0.04	16	16
allantoin	1.10	0.06	12	12
alanine & sarcosine	-1.15	0.08	16	16
2-deoxyribose 1- & 5- phosphate	1.12	0.10	8	8
N-acetylornithine	-1.38	0.11	12	12
UDP (+)	-1.04	0.11	12	12
allantoate & carbamoyl aspartate	1.24	0.11	12	12
dihydroorotate	-1.04	0.12	12	12
leucine & isoleucine	-1.17	0.12	16	16
N-carbamoylaspartate	-1.03	0.13	16	16
aspartate	-1.13	0.14	15	15
N-acetylglutamate	-1.25	0.16	8	8
lysine (b)	1.23	0.17	15	14
acadesine	-1.13	0.18	12	12
asparagine	1.07	0.18	16	16
valine	-1.14	0.19	16	16
citrulline	-1.19	0.19	16	16
UDP-glucose	-1.07	0.20	12	12
citrate	-1.01	0.22	12	12
citrate & isocitrate	-1.03	0.22	12	12
ADP (+)	1.05	0.22	10	10

Table 9.1 Continued

Metabolite	Fold change in LuxS Mutant Relative to WT	p value	WT Observati ons	LuxS⁻ Observava tions
threonine	1.01	0.23	16	16
glutamine	-1.01	0.24	16	16
UDP-glucuronate & UDP- galacturonate	-1.19	0.25	10	10
2-keto-D-gluconate	1.21	0.25	11	12
riboflavin	-1.01	0.25	12	12
tyrosine	1.11	0.26	16	15
pentose 5-phosphate*	1.15	0.26	11	12
fumarate, maleate & iso- ketovalerate (a)	1.12	0.26	10	10
glucono-1,5-lactone 6- phosphate	1.22	0.26	12	12
S-adenosyl-L-methionine (SAM)	1.14	0.26	12	12
cis- & trans-aconitate	1.06	0.26	8	8
UTP	1.04	0.26	10	10
nicotinate	1.17	0.26	8	8
thymine	1.10	0.27	10	10
4-hydroxybenzoate	1.14	0.27	10	10
fructose 1,6-bisphosphate	-1.26	0.27	12	12
glutathione	-1.04	0.27	16	16
UDP-N-acetylglucosamine	1.14	0.29	12	12
2-oxoglutarate & phenylpropionate	1.04	0.30	8	8
arginine	-1.14	0.31	14	14
gluconate	1.21	0.32	8	8
NADP+	-1.03	0.32	5	7
proline	-1.00	0.33	16	16
4-hydroxyphenylpyruvate	1.03	0.33	10	10
phenylpyruvate	1.41	0.35	12	12
malate	-1.01	0.35	12	12
ATP and dGTP	1.07	0.35	12	12
fumarate, maleate & iso- ketovalerate (b)	1.10	0.35	10	10
lactate	1.14	0.35	6	6
sedoheptulose 7-phosphate	1.16	0.35	12	12
quinolinate	1.13	0.36	8	8
O-acetylcarnitine	-1.10	0.36	12	12
hexose phosphate*	-1.09	0.38	6	6
dCTP	1.18	0.38	10	10
lysine (a)	-1.02	0.41	16	16
oxaloacetate	1.19	0.41	8	8

Table 9.1 Continued

Metabolite	Fold change in LuxS Mutant Relative to WT	p value	WT Observati ons	LuxS⁻ Obserava tions
N-acetylputrescine & pipercolate	-1.06	0.42	12	12
5'-methylthioadenosine	1.08	0.42	16	16
UDP (-)	-1.04	0.42	8	8
gluconate 6-phosphate	1.12	0.43	12	12
AMP	-1.09	0.43	12	12
5-phosphoribosyl diphosphate	-1.11	0.44	12	12
dihydroxybenzoate	1.02	0.44	8	8
phosphoenolpyruvate	1.05	0.45	12	12
3-phosphoserine	1.17	0.45	12	12
shikimate 3-phosphate	-1.05	0.45	12	12
myo-inositol	1.20	0.45	8	8
2,3- & 1,3- diphosphoglycerate	1.03	0.46	10	10
glycerol 3-phosphate	-1.15	0.46	12	12
succinate & methylmalonate	1.06	0.47	12	12
glucosamine	-1.06	0.47	12	12
dTDP	1.01	0.48	10	10
dCDP	1.08	0.49	12	12
nicotinamide	-1.07	0.49	11	12

metabolite pool labeled and unlabeled. These fractions were averaged over experiments and plotted against time. Differences were observed in 6 metabolites, hexose-phosphate, glyceraldehyde-3-phosphate, glycerate-3-phosphate, phosphoenolpyruvate, SRH, and glutathione disulfide (GSSG) (Figure 9.4).

As expected, the concentration of the LuxS substrate, SRH, was measured to be up dramatically in both positive (8.4) and negative (37.8) mode. The inconsistency between the two measurements can be explained by higher background interference in the positive mode SRM. Likewise, in negative mode carbon labeling of the SRH pool was slowed in the *luxS* mutant, but less so in positive mode. This result was even more dramatic for nitrogen flux. Therefore, the *luxS* mutant does not display significant negative feedback regulation up stream of SRH, and therefore SRH is allowed to accumulate.

The pool of glyceraldehyde-3-phosphate was decreased by 2.4 fold in the LuxS mutant. Several of the glycolysis intermediates displayed a corresponding increase in turnover rate where the LuxS mutant labeled in 5 min, while the wild type took up to 15 min to label completely in hexose-phosphate, glyceraldehyde-3-phosphate, glycerate-3-phosphate, and phosphoenolpyruvate. Thus, while there is a small change in homeostasis, the total carbon output of this pathway remains the same.

A metabolic model explaining the remainder of the changes must account for the new source of sulfur and the homocysteine portion of SRH. The ribose portion on the other hand is normally lost as DPD in the wild type strain as well. SRH is 37.8 times higher in the mutant than in the wild type. A 2010 article measuring activated methyl cycle components alone, measured the intracellular concentration of SRH to be 18.7 μM .²²⁷ Based on another study of major metabolite concentrations in *E. coli*,⁸² this concentration means SRH normally makes up 0.08% of the sulfur pool. This metabolic disruption would account for ~3% of the sulfur pool. Flux measurements show that 60% of the SRH pool is replaced over the course of an hour, leading to a roughly 1.8% drain on the normal sulfur pool.

Any metabolic disruption because SRH is not converted to homocysteine can be accounted for by the recycling of glutathione disulfide (GSSG) to cysteine (Figure 9.5). The pool of glutathione disulfide typically makes up about 19% of the sulfur pool in *E. coli*. A 1.76 fold reduction in this pool due to recycling is equal to an 8.2% shift in the sulfur pool. This shift is large enough to account for a 1.8% disruption. The remainder we

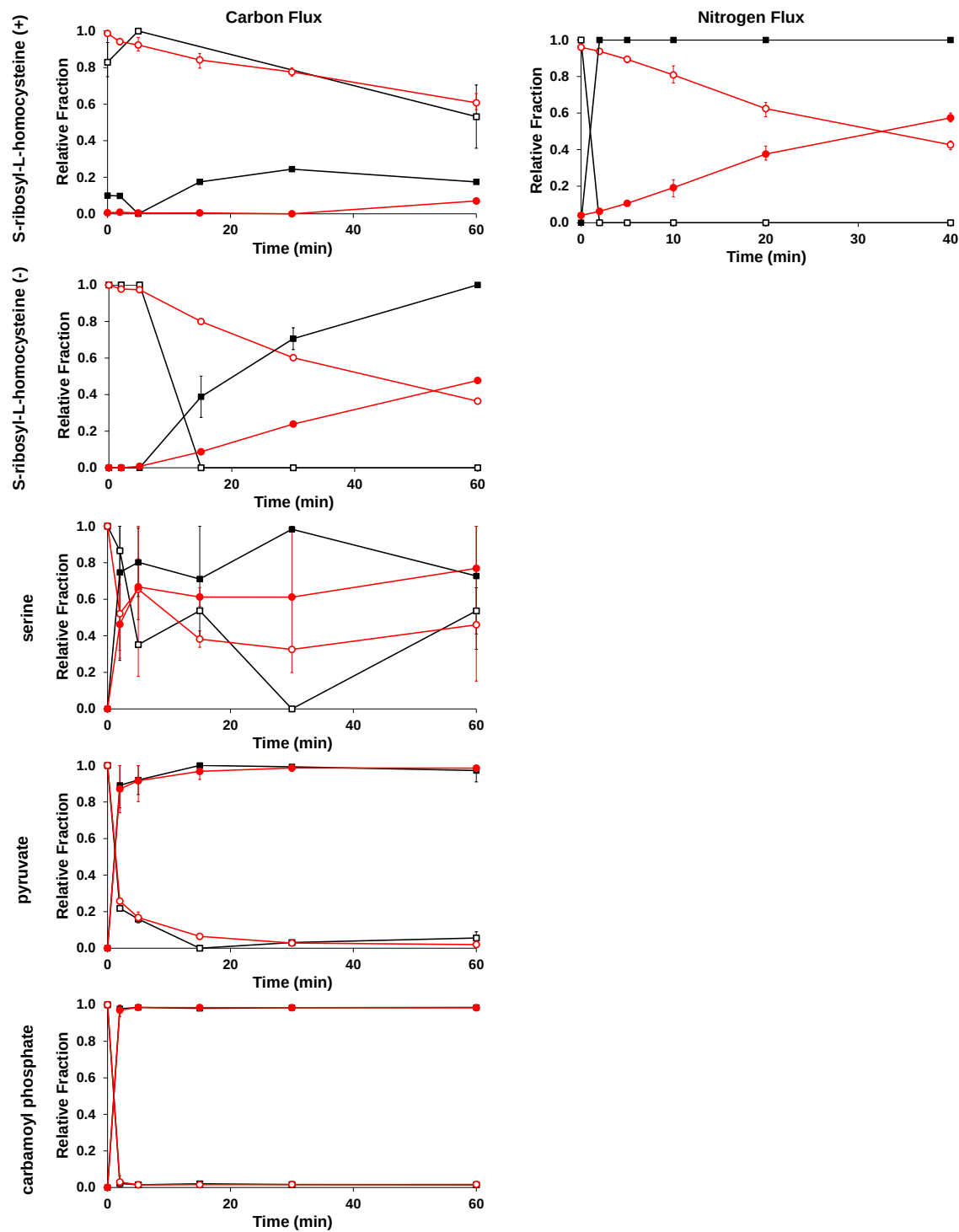


Figure 9.4. Carbon and nitrogen flux through metabolite pools. Relative fraction of unlabeled (unfilled) and fully labeled (filled) metabolites in WT (■) and *luxS*⁻ (●) *E. coli*. Partial labeling was observed in some metabolites, and therefore values may not total to 1. Measurements are an average of 4 experiments, including 2 biological replicates. Error bars represent the range of the data.

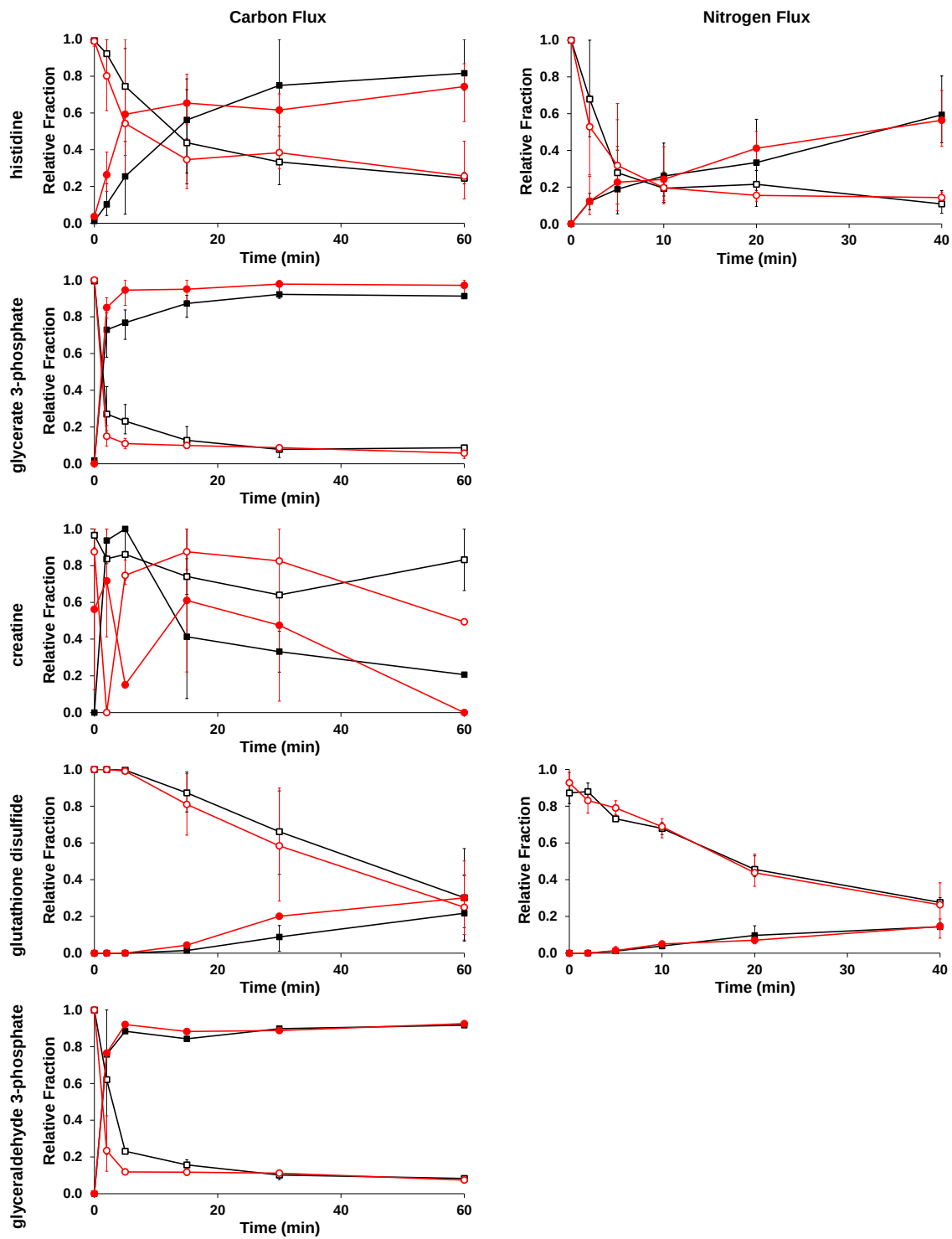


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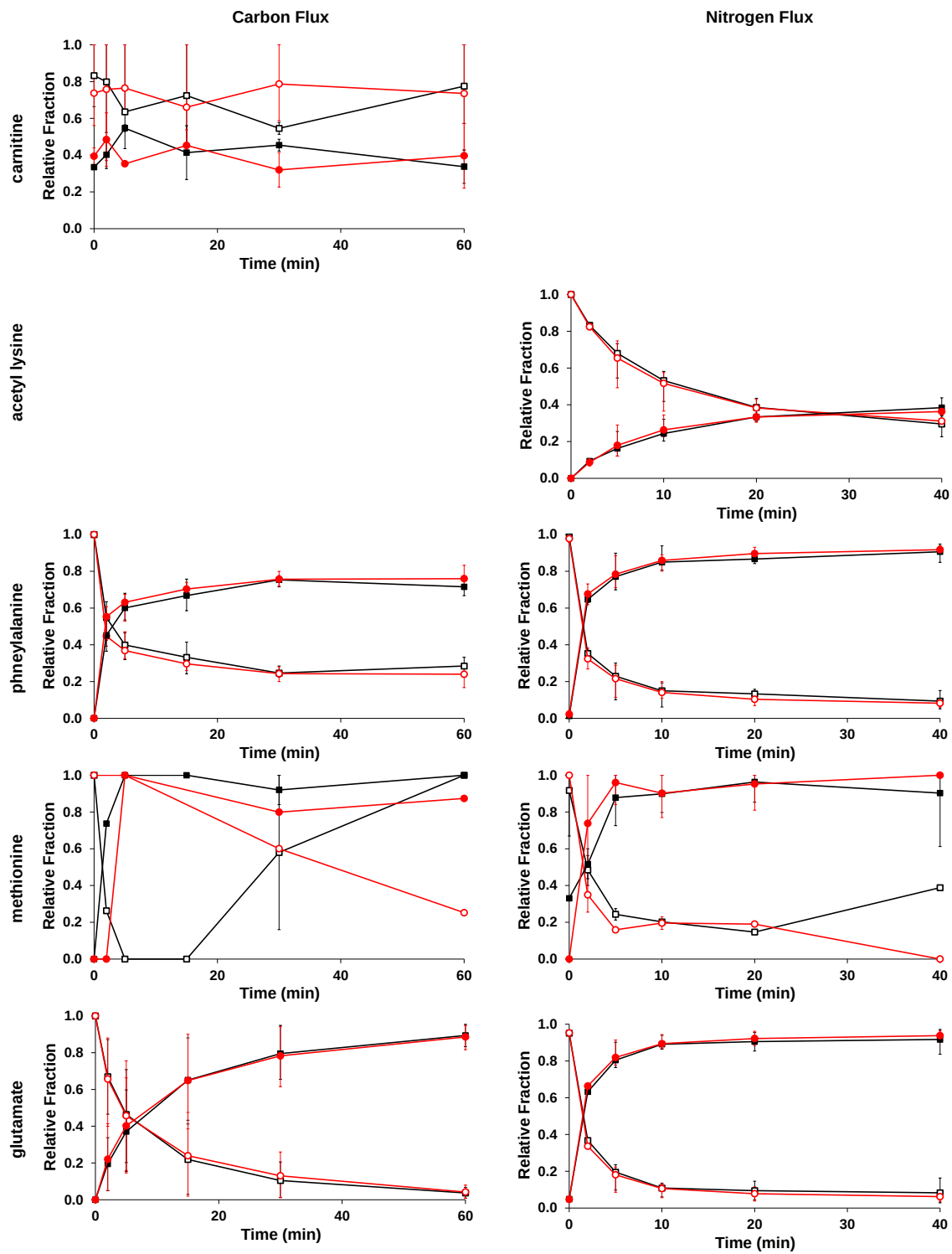


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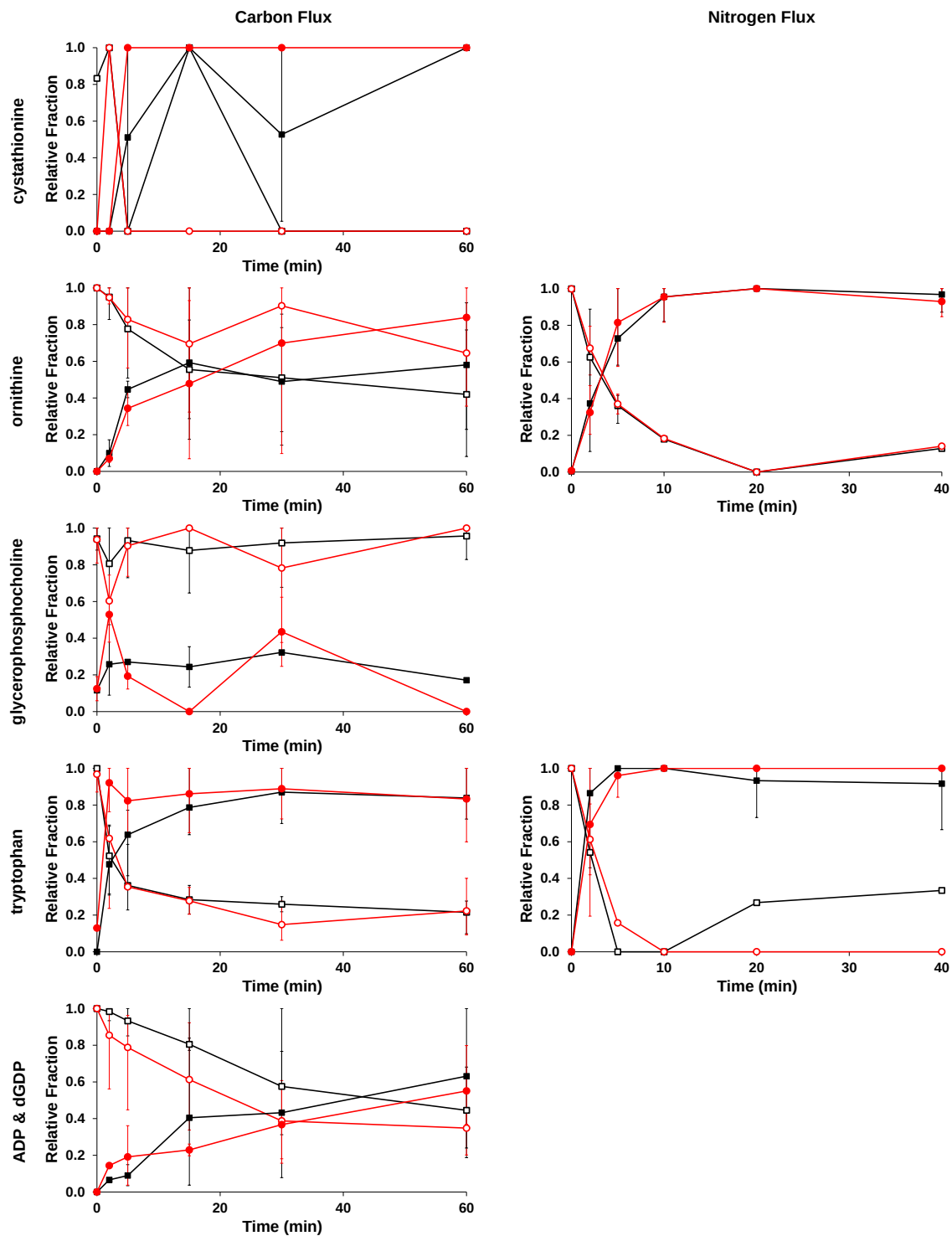


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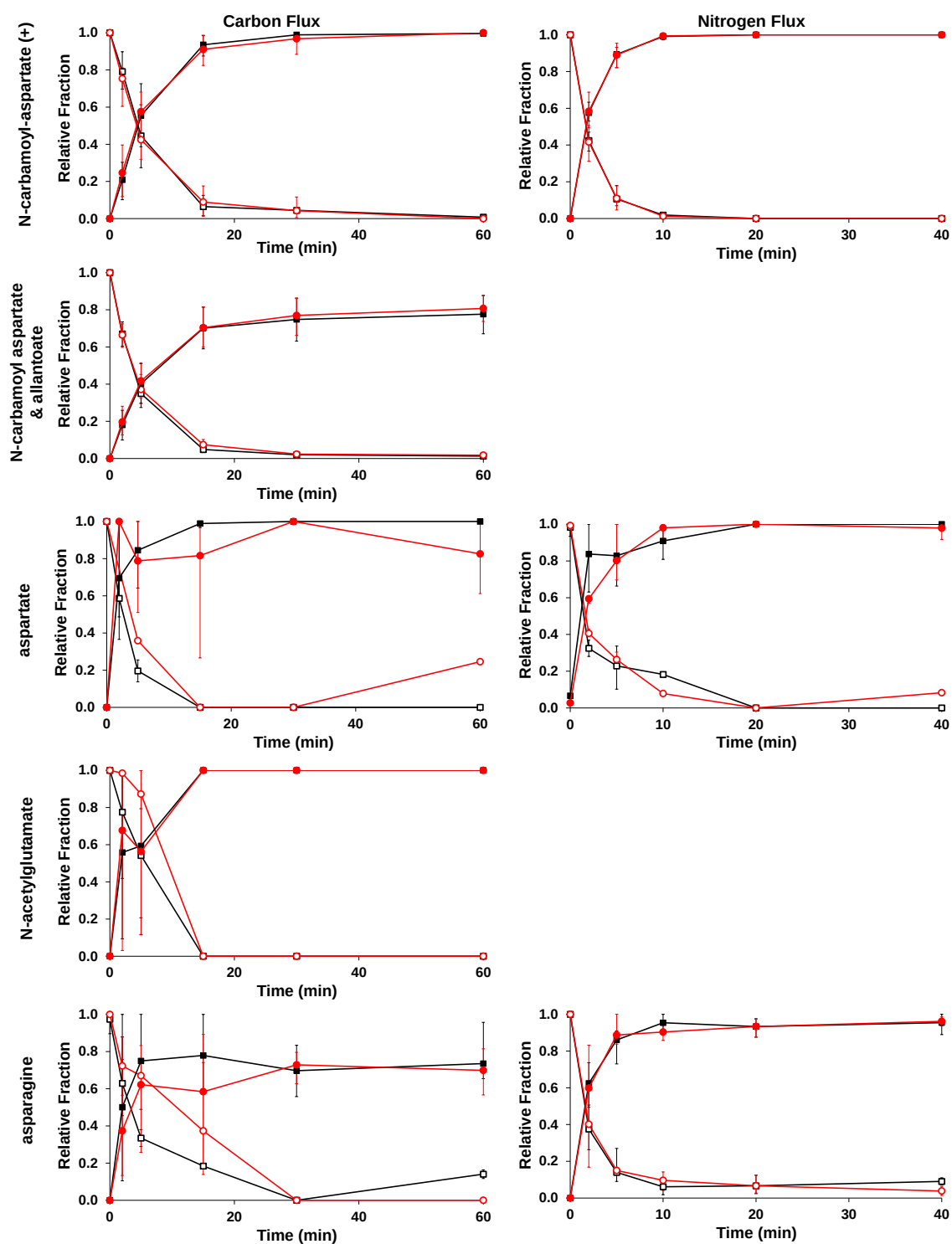


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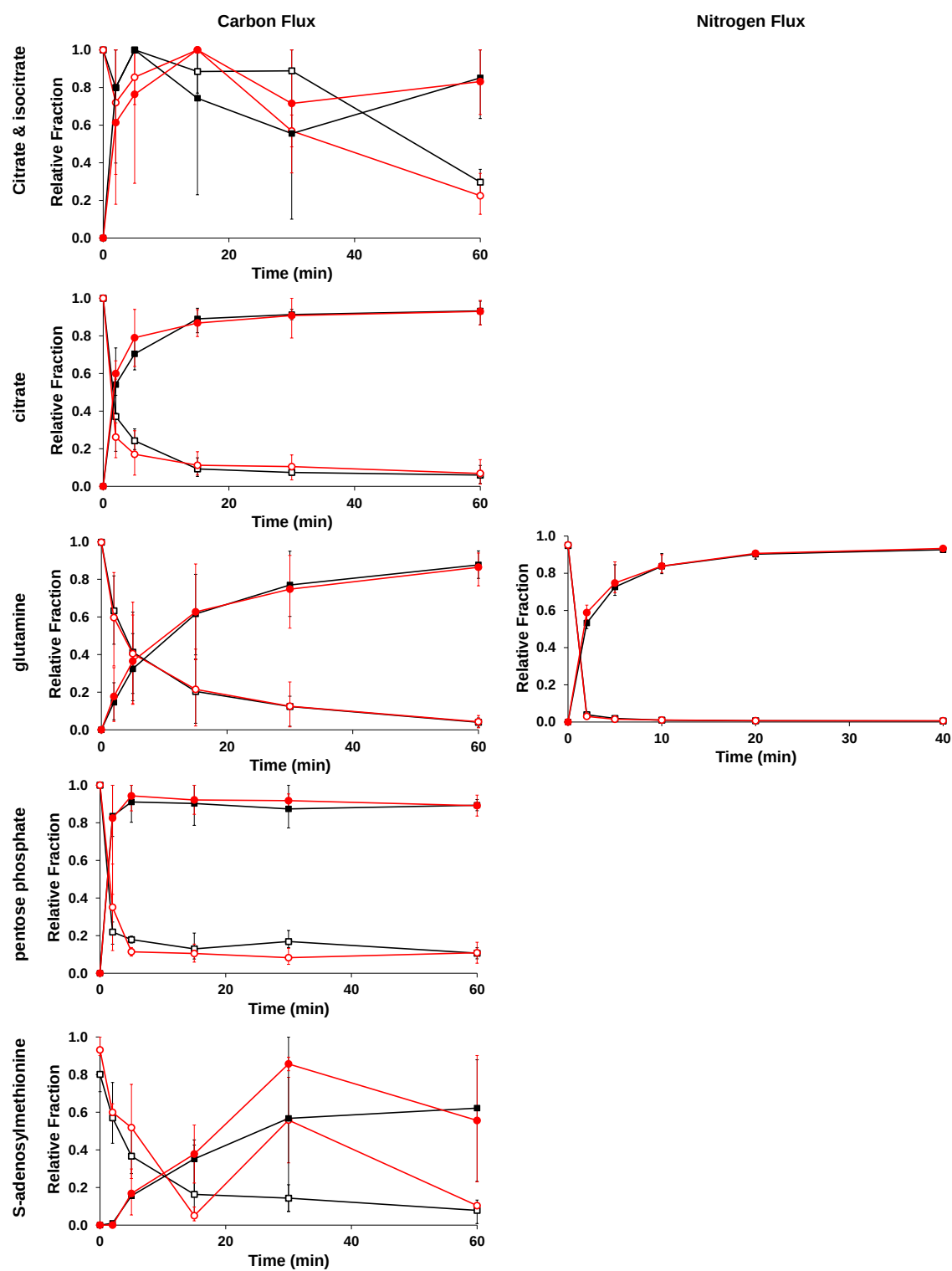


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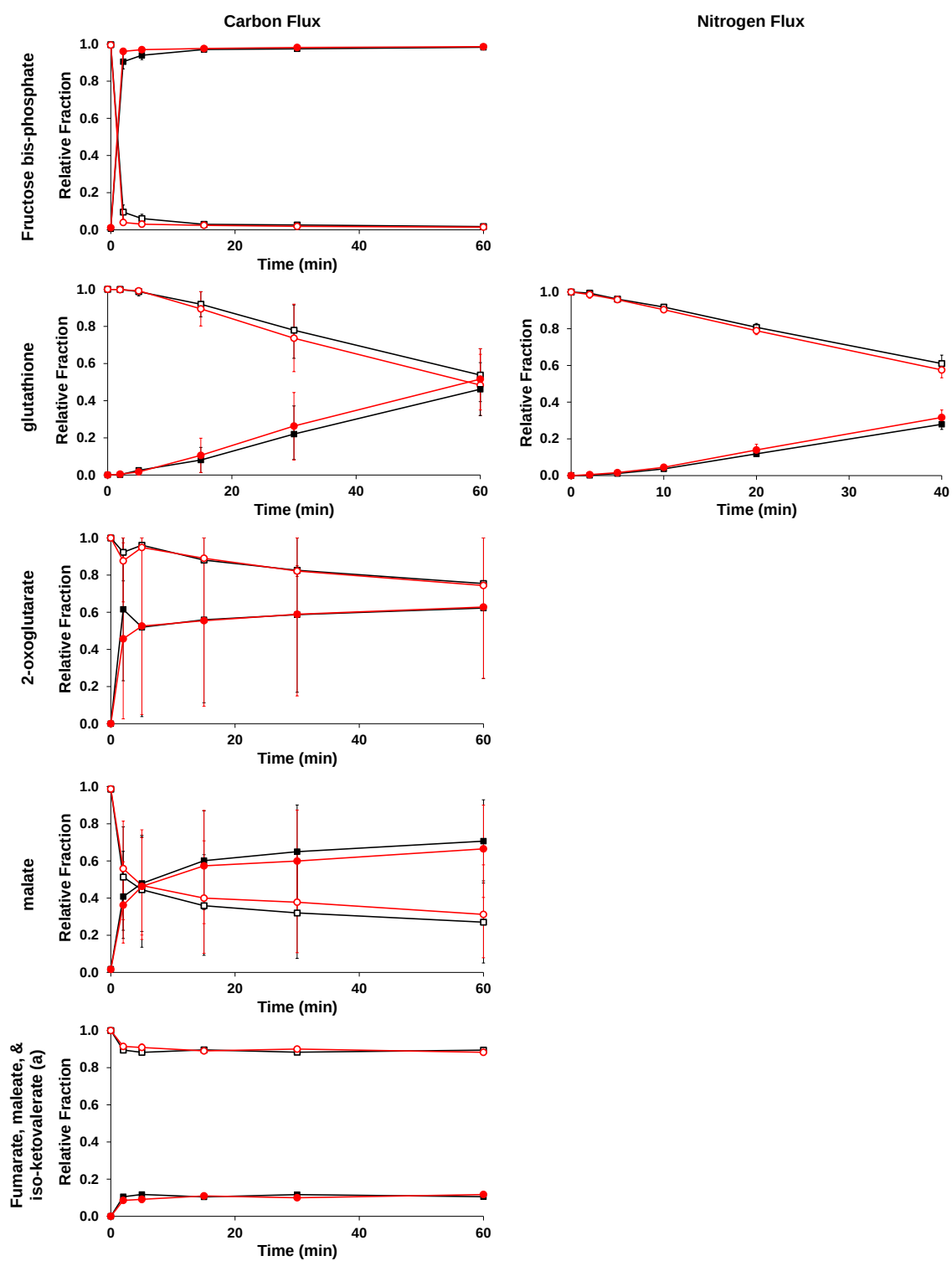


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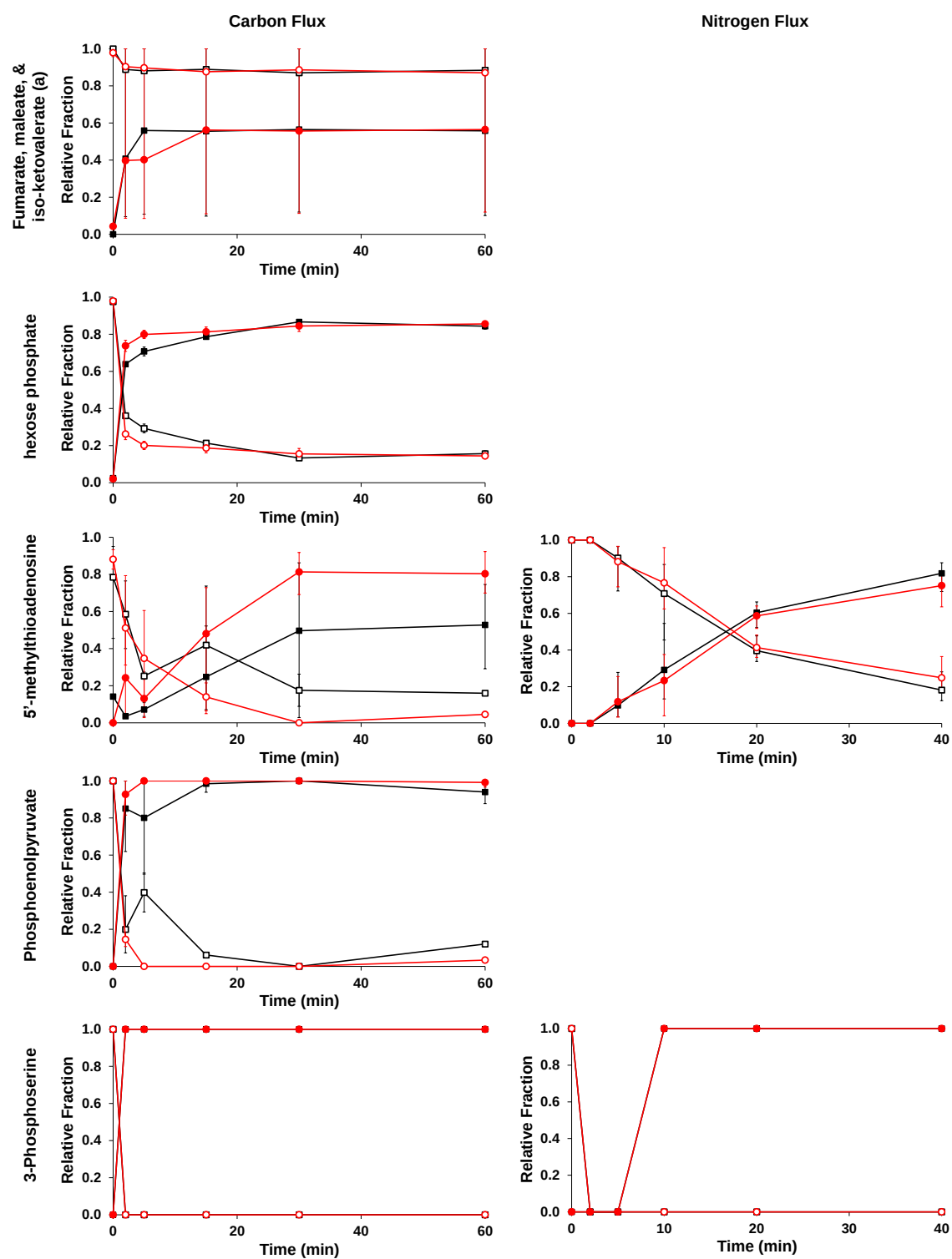


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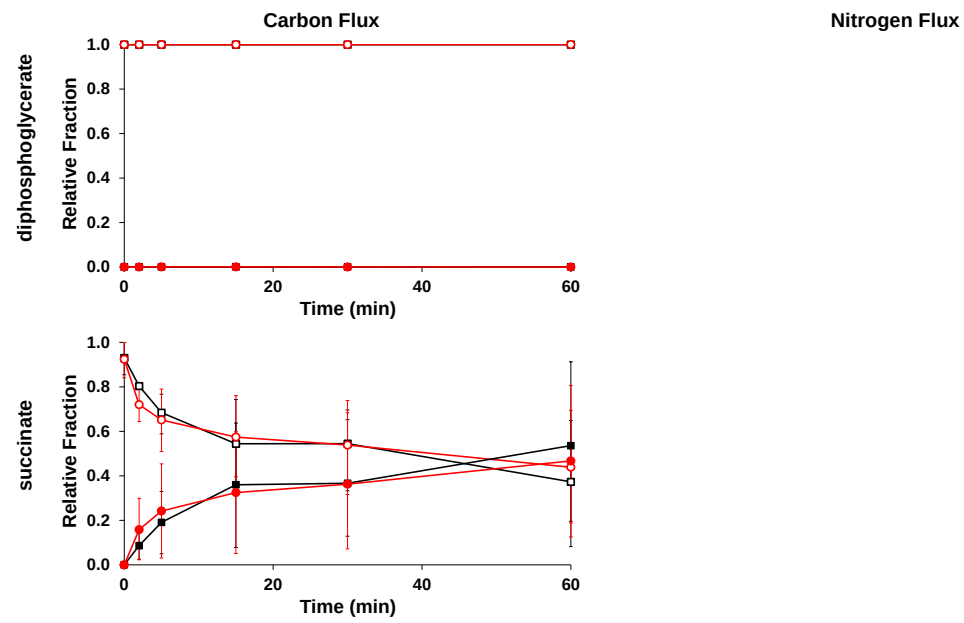


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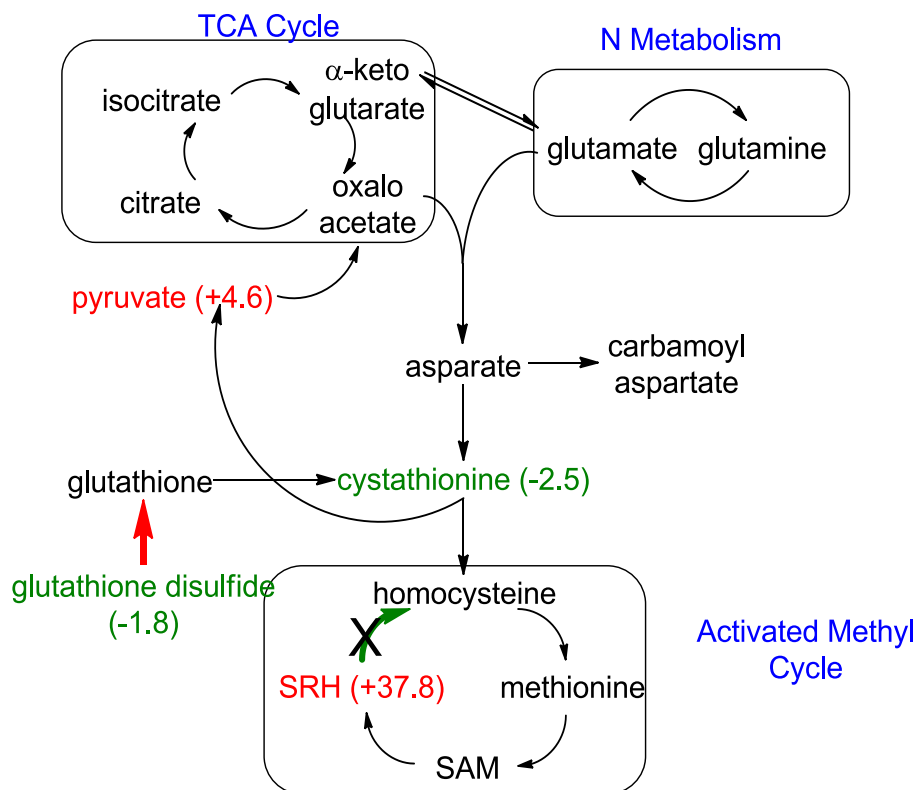


Figure 9.5. Metabolic model demonstrating how glutathione disulfide can be recycled to mitigate the effects of a *luxS* mutation.

believe is due to a decrease in oxidative stress normally triggered by DPD (see Section 9.6 for further discussion). Cysteine can be converted to cystathionine and then cleaved to homocysteine, which feeds the activated methyl cycle (AMC), and pyruvate, for which the concentrations were observed to be 4.5 times higher. Conspicuously absent from this discussion are the concentrations of other AMC components or biosynthetic precursors. These metabolites are difficult to measure by unaided ESI-MS, and the 2010 article mentioned above²²⁷ required the development of a derivatization method to quantitate them.

9.4 Comparative Nutritional Requirements

Several other experiments were completed in order to support the results of the metabolomics experiments, some of which have been reported in the literature

previously. Because we had observed a shift in homeostasis in the glycolysis intermediates, we investigated whether the strains would display a difference in glucose uptake. For this experiment the strains were grown in liquid culture on minimal media that contained 0.4% w/v glucose and both optical density and [glucose] were monitored over 20 h. A small difference in timing was observed over the decline from 6-9 h. However when the data was plotted against optical density instead to account for the slightly lower cell density in the *luxS*⁻ culture, the strains were not different in rate of glucose consumption (Figure 9.6). The small differences in glycolysis therefore were not due to a difference in glucose consumption or increased nutrient demand in the mutant.

Demands for carbon, nitrogen and sulfur were also investigated. Wild type and *luxS*⁻ strains of *E. coli* were inoculated into minimal media, or minimal media without key nutrients. Growth was then observed by optical density over 9 h (Figure 9.7). Surprisingly, growth in sulfur starvation conditions was identical for both strains and was comparable to normal minimal media. Slower growth was observed for both strains in nitrogen starved cultures, and no growth was observed carbon starved cultures. These results taken together with the glucose consumption and metabolomics experiments, show that there is little reason to believe that a mutation in *luxS* causes global metabolic disregulation that would evolutionarily require maintenance of the gene over a more efficient version of the AMC.

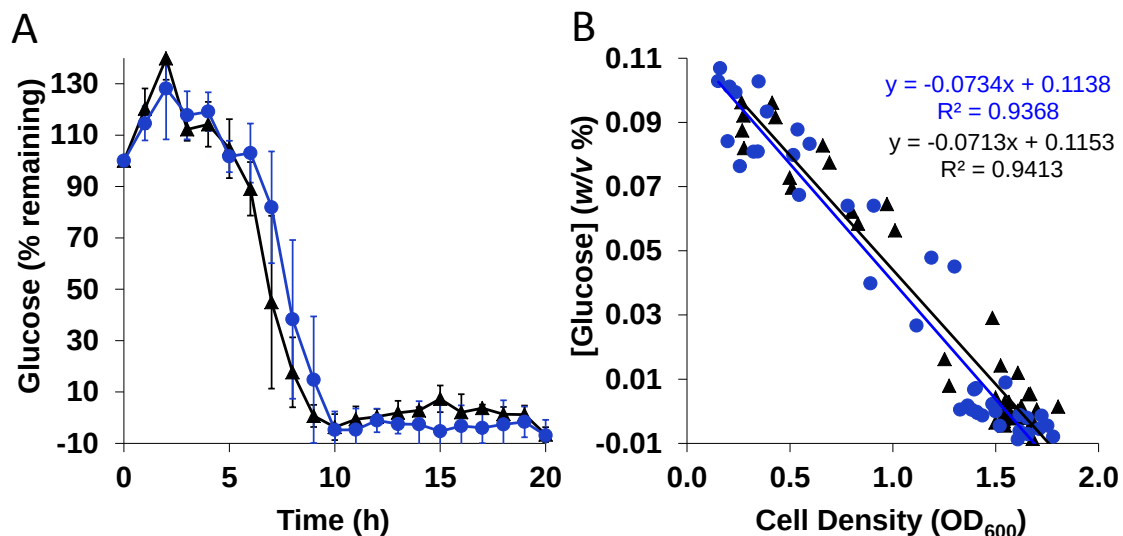


Figure 9.6. Glucose uptake in *E. coli* BW25113 (▲) or *luxS*⁻ mutant JW2662-1 (●) when grown in liquid culture on minimal media.

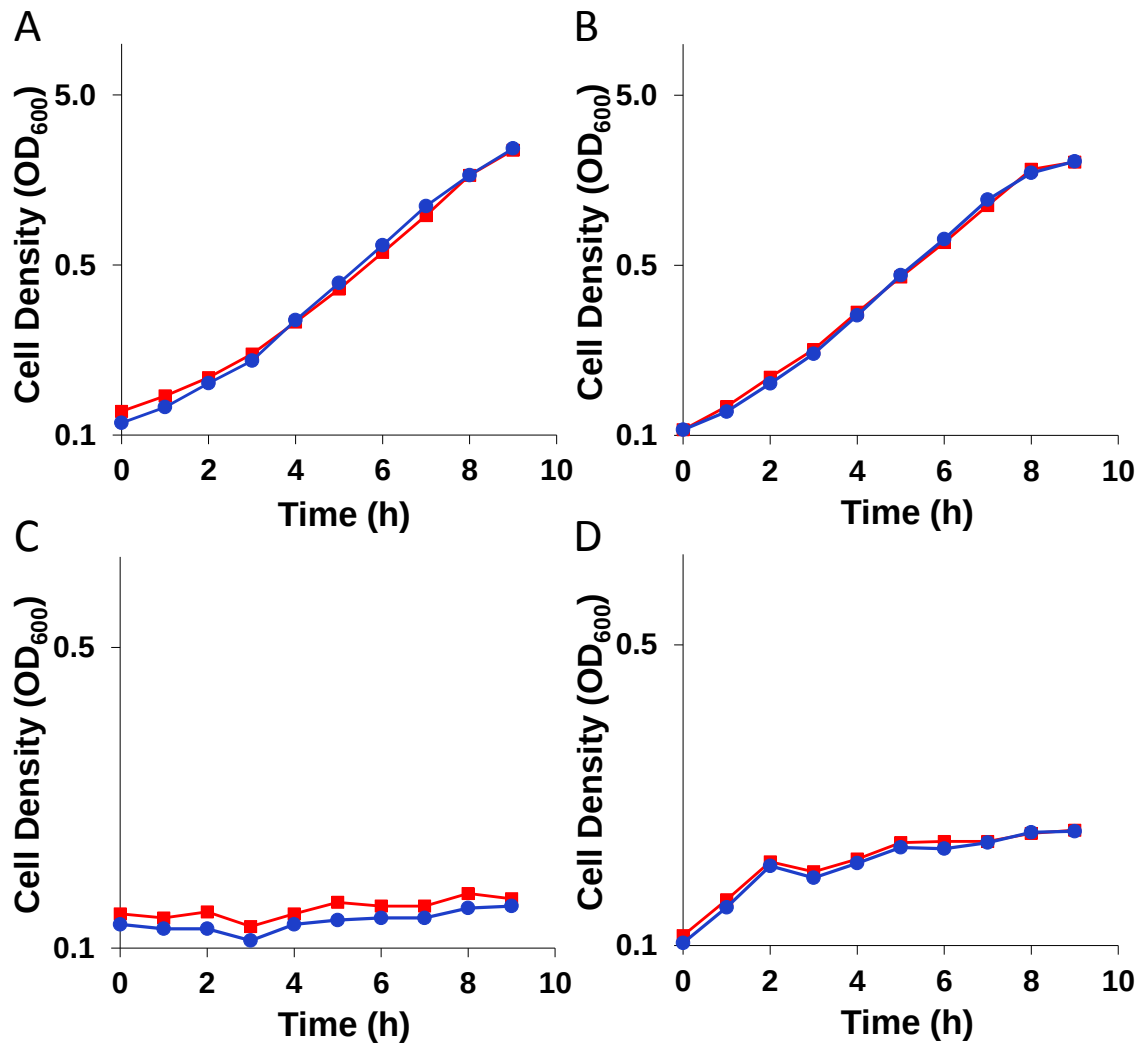


Figure 9.7. Growth of *E. coli* BW25113 (■) or *luxS*⁻ mutant JW2662-1 (●) when grown in liquid culture on A) minimal media, B) minimal media without a sulfur source, C) minimal media without a carbon source, and D) minimal media without a nitrogen source.

Experiments exploring whether *E. coli* could grow on DPD were completed as well. DPD is synthesized with the aid of a cyclohexylidene protecting group, which is removed with sulfuric acid immediately prior to use, and no further purification is performed. Therefore all DPD solutions contain equimolar amounts of cyclohexanone, and acidic cyclohexanone is used in any controls for DPD. A culture of BW25113 was washed with minimal salts before inoculation at 0.2 OD₆₀₀ into 10 mL minimal salts containing 2.35 mM DPD or 2.35 mM cyclohexanone and 0.1% v/v H₂SO₄. No DPD consumption nor growth was observed over 3h, rather the OD₆₀₀ decreased over time. No evidence that *E. coli* could use DPD as a carbon source was observed (Figure 9.8).

9.5 Host Immune Response to AI-2

Having disposed of the metabolism hypothesis, we returned to the defense mechanism hypothesis. There are three ways DPD consumption could serve a defensive purpose for the organism. First, and related to the metabolism question, if DPD were toxic, export of DPD to prevent the accumulation of high concentrations would prevent some toxicity, while slow re-uptake later would prevent waste of a valuable carbon source. This hypothesis can largely be discarded on the basis of experiments already discussed. Because *E. coli* has a means to correct for any metabolic losses, there is no reason the bacteria would have to make a “toxic” metabolite. Importation occurs quickly over at

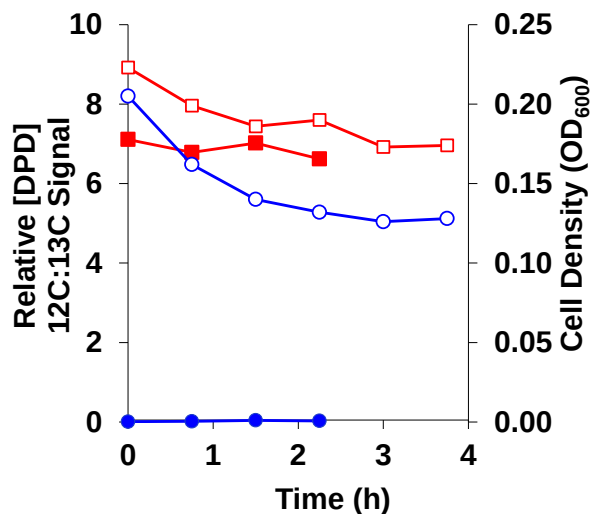


Figure 9.8. Growth (open) and DPD consumption (filled) of *E. coli* grown in minimal salts (●) or minimal salts with 2.35 mM DPD (■).

most 1.5 h, which is not in agreement with the slow re-uptake explanation. Second, the bacteria could import the molecule to confuse the communication of invading species. While we have not ruled out this possibility, we do know that the commensal species would need to maintain the Lsr transporter instead of responding to a signal. This is because only high concentrations ($> 50 \mu\text{M}$) trigger early importation. Finally, the bacteria could be preventing a host response by removing an antigen from the extracellular matrix.

A mouse macrophage cell line (RAW264.7) was chosen for experiments probing the host immune response to DPD. We expected that a host response to a unique signal of bacterial origin would be through the transcription factor NF- κ B. Responses to classical bacterial antigens such as lipopolysaccharide (LPS) are routed through NF- κ B signaling leading to the transcription of cytokines TNF- α , IL-1 β and IL-6.^{228,229} The AHL class of quorum sensing signals is known to modulate the immune system by binding the peroxisome proliferator activated receptors (PPARs). The AHL can act as either an agonist or an antagonist depending on the PPAR isoform, which in turn modulates the NF- κ B, MAPK p38, and JAK/STAT pathways, all of which play a role in the immune response.²³⁰ Suggestively, a study of *Vibrio vulnificus* quorum sensing mutants showed that a *luxS* mutation caused the largest delay or decrease in total expression of TNF- α , IL-1 β , IL-6 and NO in RAW264.7 cells.²³¹ Although, this phenomenon may be attributed to a disruption of quorum sensing causing the inhibition of virulence factor production. Further, small molecules with the same α -diketone functional group as DPD, methylglyoxal and glyoxal, cause an increase in IL-8 and IL-6 secretion in an intestinal cell line, Caco-2.²³² The typical immune response to bacteria and viruses are different, and often promotion of one response leads to the inhibition of another.²³³ To control for this possibility one of the virally promoted cytokines, IFN- α was included in the cytokine panel.

RAW264.7 cells were plated at 5×10^5 cells/mL in a 48 well plate and allowed to attach for 6 h. The cells were then stimulated with varying concentrations of DPD or a control solution containing cyclohexanone and acid for 18 h. Half of the treated cells also received 95 ng/mL LPS. The following day, supernatants were collected for ELISA analyses, and the cell pellets saved for RNA extraction. In contrast to our initial hypothesis, DPD actually lowered both TNF- α (34%) and IL-6 (77%) production in LPS treated cells at 188 μM DPD (Figure 9.9). A decrease was also observed for TNF- α

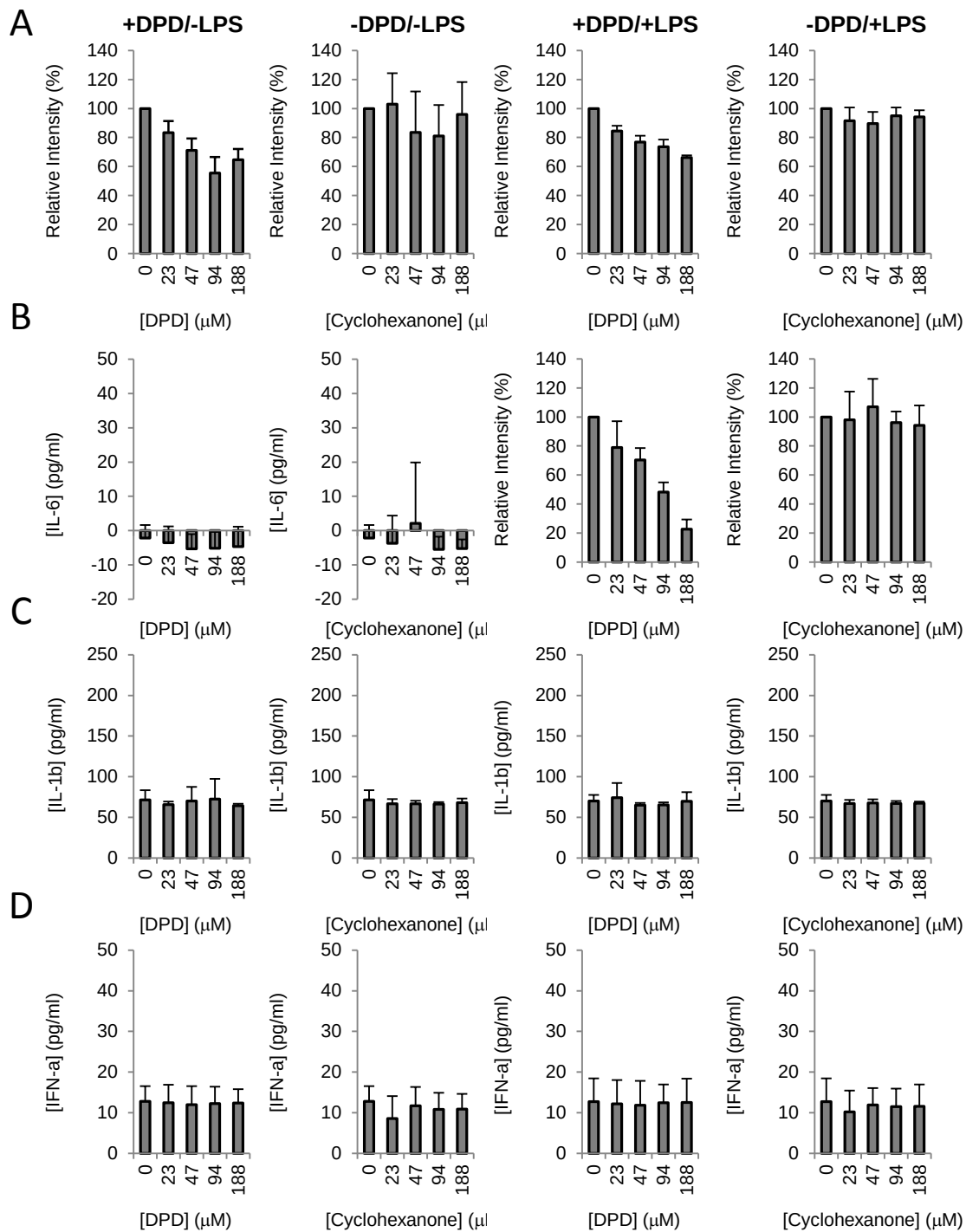


Figure 9.9. Concentration or relative intensity of immune response markers A) TNF- α , B) IL-6, C) IL-1 β , D) IFN- α , and E) NO when RAW264 cells were stimulated for 18 h with DPD and/or LPS.

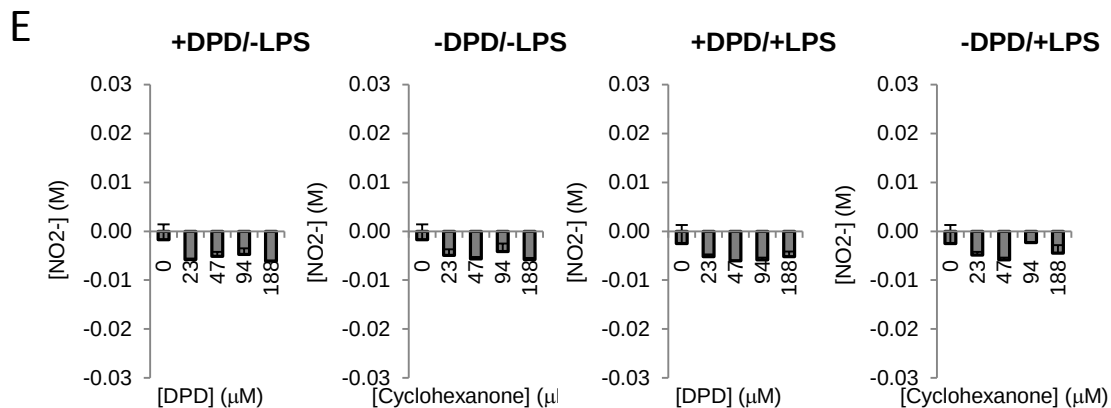


Figure 9.9. Continued.

(35%) in cells not treated with LPS, but basal production levels were too low to observe a difference in IL-6. Production of IL-1 β and IFN- α were not observed in the assays, likely due to an inadequate LPS concentration. Similarly NO levels were not above the limit of detection as measured by the Griess assay.^{234,235} AnnexinV-PI labeling²³⁶ of the treated cells followed by flow cytometry, showed no signs of cell death due to any of the treatments (Figure 9.10).

Because there are no other labs with the technology necessary to quantify [DPD] in complex media, little is known about the biologically relevant concentrations. To begin addressing this question, C57BL/6J male mice fed either a low (10%) or high fat diet were sacrificed, and four segments of the intestinal tract were harvested: stomach, 3 cm of small intestine starting from the distal sphincter of the stomach, the cecum and the descending colon. The contents of each segment were derivatized, and the [DPD] was measured (Figure 9.11). Concentrations ranged from 20-300 pmol DPD / g of contents; however, these results are difficult to quantitate because the efficiency of recovery is likely to vary between segments. Moreover, the local concentration is more physiologically important than the bulk concentration. There is therefore inadequate evidence to determine if 188 μ M DPD is a biologically relevant concentration. On the other hand, glucose concentrations in the intestinal lumen²³⁷ range from 1 to 25 mM and are high enough to produce 150 μ M concentrations in *E. coli* under laboratory conditions (see section 8.2).

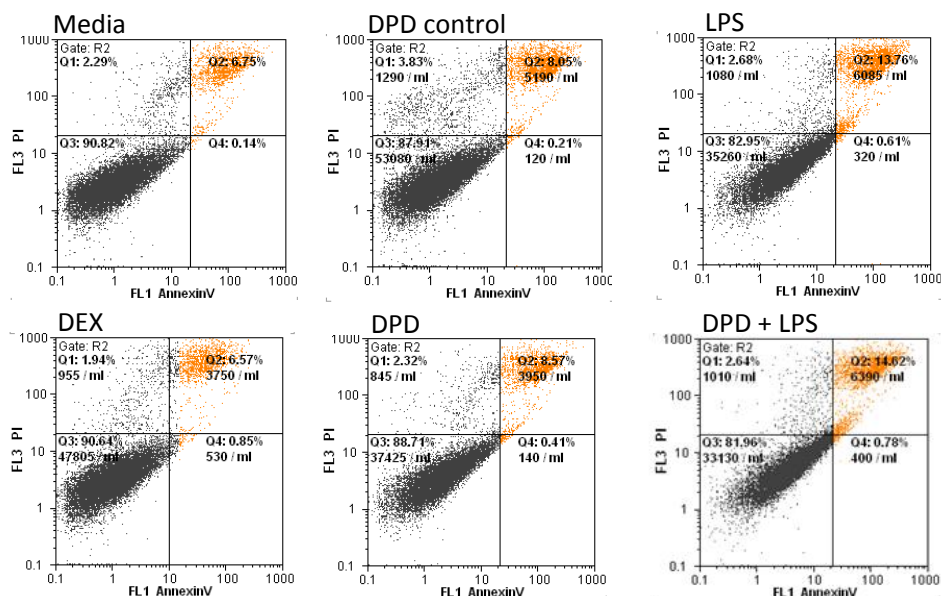


Figure 9.10. AnnexinV-PI labeling of RAW264 cells, analyzed by flow cytometry showed no signs of cell death in any of the treatments. DPD Control = 50 μ M cyclohexanone DPD = 50 μ M, LPS=100 ng/mL, and DEX = 100 nM. Data are representative of two experiments.

Epinephrine, a host produced signal, was previously reported to decrease *IsrB* gene expression by 7 fold.²³⁸ If a link between epinephrine and extracellular AI-2 concentrations could be established, this would build further evidence of a host-bacteria relationship mediated by AI-2. For these purposes, wild type, *luxS*⁻ (negative control), and *IsrB*⁻ (positive control) strains of *E. coli* were treated with epinephrine and observed over the relevant hours (3-8 h) of peak DPD production. A 28% increase in [DPD] was observed in the wild type in the first experiment. However we were unable to reproduce this result.

9.6 Global Macrophage Response to AI-2

While the limitations of transcriptomics have already been discussed, it was chosen to build upon the ELISA data. RNA could be isolated from the same macrophages as were used for the cytokine assays, and as an established technique the work could be outsourced fairly inexpensively for the amount of data gathered. This indeed was the path chosen, and microarrays were completed for 7 treatments, 5 of which were

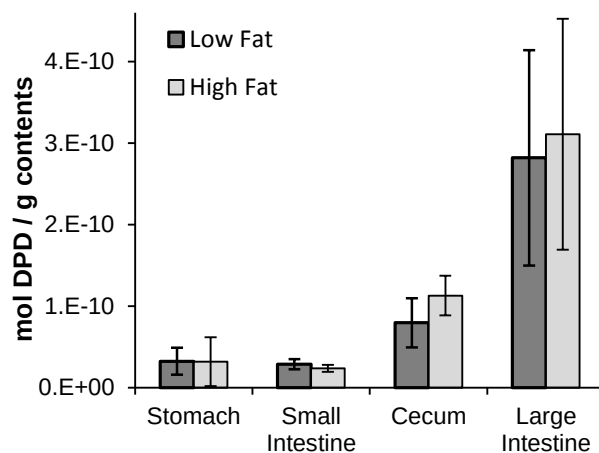


Figure 9.11. [DPD] measured from the contents of several segments the GI tract of male C57BL/6J mice fed either a low fat (dark) or high fat (light) diet. Values are the average of six mice, and error bars indicate a standard deviation. Chow was measured in duplicate and the error bars represent the range.

done in duplicate: RPMI, 94 μ M DPD, 94 μ M cyclohexanone, 94 μ M DPD + 95 ng/mL LPS, 94 μ M cyclohexanone + 95 ng/mL LPS, 47 μ M DPD + 95 ng/mL LPS, and 23 μ M DPD + 95 ng/mL LPS.

The results indicate there were no significant differences between the media control and cyclohexanone treatment and no differences between the three DPD + LPS treatments. When 94 μ M DPD treatment was compared to 94 μ M cyclohexanone 106 probes were found to be expressed significantly differently (Table A9.1). These primarily fell into three categories: oxidative stress and redox chemistry, immune response, and cation binding (Tables 9.2, 9.3, and 9.4 respectively). The 34 probes that were significantly different between 94 μ M DPD + 95 ng/mL LPS, 94 μ M cyclohexanone + 95 ng/mL LPS also fell into these categories. The 106 probes significantly different in the 94 μ M DPD treatment - 94 μ M cyclohexanone pair were also compared to the 716 expressed differently in the 94 μ M cyclohexanone-94 μ M cyclohexanone + 95 ng/mL LPS pair. Of these, 25 probes were shared between the two groups and 4 were in opposite directions (Table 9.5).

In general, oxidation related genes are expressed at a higher level, and immune system related genes are decreased. Most interesting among these data are the shared link between the three over-arching themes. The top two major changes in expression

Table 9.2. Oxidative Stress and Redox

Gene Symbol	Gene Name	DPD - CH		DPD + LPS – CH +LPS	
		Fold Change	p-value	Fold Change	p-value
ABCB6	ATP-binding cassette, sub-family B, member 6	1.79	0.0256		
AKR1A4	aldo-keto reductase family 1, member A4	1.57	0.0254		
AKR1B8	aldo-keto reductase family 1, member B8	3.21	0.0014	2.51	0.0150
ALDH2	aldehyde dehydrogenase 2, mitochondrial	1.46	0.0488		
ALOX5AP	arachidonate 5-lipoxygenase activating protein	1.72	0.0124	1.58	0.0731
		1.84	0.0415		
BLVRB	biliverdin reductase B	4.37	1.4E-05	2.63	0.0005
		5.25	0.0008	3.10	0.0122
CAT	catalase	3.99	0.0011	2.36	0.0258
CBR3	carbonyl reductase 3	3.66	0.0014	2.38	0.0259
		3.87	0.0048	3.19	0.0254
CYB5	cytochrome b-5	2.63	0.0017	2.30	0.0122
		3.57	0.0085		
G6PDX	glucose-6-phosphate dehydrogenase X-linked	2.21	0.0078	1.74	0.0838
GCLM	glutamate-cysteine ligase, modifier subunit	7.57	1.4E-05	4.22	0.0004
GSS	glutathione synthetase	1.53	0.0085	1.51	0.0808
		1.56	0.0102	1.42	0.0479
GSTA3	glutathione S-transferase, alpha 3	12.68	0.0793		
HMOX1	heme oxygenase (decycling) 1	2.28	0.0493	2.72	0.0416

Table 9.2. Continued

Gene Symbol	Gene Name	DPD - CH		DPD + LPS – CH +LPS	
		Fold Change	p-value	Fold Change	p-value
IDH1	isocitrate dehydrogenase 1 (NADP+), soluble	1.81	0.0666		
MGST1	microsomal glutathione S-transferase 1	2.13	0.0110		
MGST2	microsomal glutathione S-transferase 2	2.63	0.0011		
PRDX1	peroxiredoxin 1; predicted gene 7204	2.14	0.0162	2.41	0.0234
SQRDL	sulfide quinone reductase-like (yeast)	2.35	0.0114		
SRXN1	sulfiredoxin 1 homolog (S. cerevisiae)	3.41	0.0002	2.38	0.0028
		4.31	0.0041	3.06	0.0348
		4.70	0.0035	3.07	0.0410

Table 9.3. Immune response

Gene Symbol	Gene Name	DPD - CH		DPD + LPS – CH +LPS	
		Fold Change	p-value	Fold Change	p-value
ALOX5AP	arachidonate 5-lipoxygenase activating protein	1.72	0.0124		
		1.84	0.0415		
CASP1	caspase 1; hypothetical protein LOC100044207	1.76	0.0066		
CCL4	chemokine (C-C motif) ligand 4	-1.88	0.0115		
		-1.83	0.0363		
CCL7	chemokine (C-C motif) ligand 7			-5.53	0.0479
CLEC4D	C-type lectin domain family 4, member d	1.64	0.0114		
CLEC4N	C-type lectin domain family 4, member n	3.53	0.0916		
CXCL16	chemokine (C-X-C motif) ligand 16	-1.55	0.0976		
CXCR4	chemokine (C-X-C motif) receptor 4	1.99	0.0056		
CYB5	cytochrome b-5	2.63	0.0017		
DHX58	DEXH (Asp-Glu-X-His) box polypeptide 58	-1.76	0.0060		
EIF2AK2	eukaryotic translation initiation factor 2-alpha kinase 2	-1.63	0.0488		
ESD	similar to esterase D/formylglutathione hydrolase	1.39	0.0288		
		3.14	0.0011		
HMOX1	heme oxygenase (decycling) 1	2.28	0.0493		
IFITM1	interferon induced transmembrane protein 1	-1.57	0.0488		
IRF9	interferon regulatory factor 9	-1.62	0.0377		
IRGM1	immunity-related GTPase family M member 1	-1.78	0.0114		

Table 9.3. Continued

Gene Symbol	Gene Name	DPD - CH		DPD + LPS – CH +LPS	
		Fold Change	p-value	Fold Change	p-value
LYZL4	lysozyme-like 4	1.57	0.0415		
OAS1G	2'-5' oligoadenylate synthetase 1G	-1.88	0.0673		
OAS2	2'-5' oligoadenylate synthetase 2	-2.04	0.0060		
		-2.04	0.0060		
PANX1	pannexin 1	1.98	0.0052		
		2.10	0.0094		
PRDX1	peroxiredoxin 1; predicted gene 7204	1.52	0.0667		
		2.14	0.0162		
RSAD2	radical S-adenosyl methionine domain containing 2	-1.76	0.0985		
SERPINB6	serine (or cysteine) peptidase inhibitor, clade B, member 6	1.61	0.0357		
SLC11A1	solute carrier family 11 (proton-coupled divalent metal ion transporters), member 1	3.28	0.0008		
		3.92	0.0012		
		4.12	0.0022		
		4.25	0.0018		
SLC40A1	solute carrier family 40 (iron-regulated transporter), member 1	5.86	0.0028		
TNFAIP2	tumor necrosis factor, alpha-induced protein 2	1.63	0.0549		
		1.71	0.0114		
TRAF1	TNF receptor-associated factor 1			-1.71	0.0995

Table 9.4. Metal Binding and Transport

Gene Symbol	Gene Name	DPD - CH		DPD + LPS – CH +LPS	
		Fold Change	p-value	Fold Change	p-value
ABCB6	ATP-binding cassette, sub-family B (MDR/TAP), member 6	1.79	0.0256		
AFG3L2	similar to AFG3(ATPase family gene 3)-like 2 (yeast);	1.37	0.0367		
CALR3	calreticulin 3	2.52	0.0011	3.09	0.0028
		3.59	0.0008	2.32	0.0048
CAT	catalase	3.99	0.0011	2.36	0.0258
CYB5	cytochrome b-5	2.63	0.0017	2.30	0.0122
		3.57	0.0085		
DHX58	DEXH (Asp-Glu-X-His) box polypeptide 58	-1.76	0.0060		
DNMT3L	similar to DNA cytosine-5 methyltransferase 3-like protein	2.59	0.0254		
		2.92	0.0114		
EMR1	EGF-like module containing, mucin-like, hormone receptor-like sequence 1	1.73	0.0358		
GSS	glutathione synthetase	1.53	0.0085	1.42	0.0479
		1.56	0.0102		
HMOX1	heme oxygenase (decycling) 1	2.28	0.0493	2.72	0.0416
ITPR2	inositol 1,4,5-triphosphate receptor 2	1.99	0.0114	1.98	0.0267
MITD1	microtubule interacting and transport, domain containing 1	-1.47	0.0294		
PANX1	pannexin 1	1.98	0.0052	1.89	0.0201
		2.10	0.0094		
PDLIM4	PDZ and LIM domain 4	2.39	0.0006		

Table 9.4. Continued

Gene Symbol	Gene Name	DPD - CH		DPD + LPS – CH +LPS	
		Fold Change	p-value	Fold Change	p-value
SLC11A1	solute carrier family 11 (proton-coupled divalent metal ion transporters), member 1	3.28	0.0008		
		3.92	0.0012		
		4.12	0.0022		
		4.25	0.0018		
SLC40A1	solute carrier family 40 (iron-regulated transporter), member 1	5.86	0.0028	3.64	0.0337
SLC7A11	solute carrier family 7 (cationic amino acid transporter, y+ system), member 11	2.24	0.0026		
SRXN1	sulfiredoxin 1 homolog (S. cerevisiae)	3.41	0.0002	2.38	0.0028
		4.31	0.0041	3.06	0.0348
		4.70	0.0035	3.07	0.0410
USE1	unconventional SNARE in the ER 1 homolog (S. cerevisiae)	-1.40	0.0377		

Table 9.5. Effects Shared by DPD and LPS

Gene Symbol	Gene Name	DPD - CH		CH – CH +LPS	
		Fold Change	p-value	Fold Change	p-value
CCL4	chemokine (C-C motif) ligand 4	-1.88	0.0115	-4.16	0.0005
NCAPH2	non-SMC condensin II complex, subunit H2	-1.50	0.0115	1.57	0.0037
USE1	unconventional SNARE in the ER 1 homolog (S. cerevisiae)	-1.40	0.0377	1.28	0.0382
ALDH2	aldehyde dehydrogenase 2, mitochondrial	1.46	0.0488	1.45	0.0166
UBE2E2	ubiquitin-conjugating enzyme E2E 2 (UBC4/5 homolog, yeast)	1.47	0.0419	-1.45	0.0155
UGT1A10	similar to UDP glycosyltransferase 1 family polypeptide A13	1.57	0.0185	1.87	0.0018
RIOK3	RIO kinase 3 (yeast)	1.62	0.0290	-2.02	0.0101
CLEC4D	C-type lectin domain family 4, member d	1.64	0.0114	-1.70	0.0039
TNFAIP2	tumor necrosis factor, alpha-induced protein 2	1.71	0.0114	-1.98	0.0017
EMR1	EGF-like module containing, mucin-like, hormone receptor-like sequence 1	1.73	0.0358	-1.60	0.0214
NRP1	neuropilin 1	1.93	0.0416	1.70	0.0376
LOC10004	NAALADL1 N-acetylated alpha-linked acidic dipeptidase-like 1 [H. sapiens]	1.93	0.0160	-2.62	0.0056
ALAS1	aminolevulinic acid synthase 1	2.07	0.0124	-1.58	0.0341
PRDX1	peroxiredoxin 1; predicted gene 7204	2.14	0.0162	-1.42	0.0374

Table 9.5. Continued

Gene Symbol	Gene Name	DPD - CH		CH – CH +LPS	
		Fold Change	p-value	Fold Change	p-value
SLC7A11	solute carrier family 7 (cationic amino acid transporter, y+ system), member 11	2.24	0.0026	-2.96	0.0005
LOC22416	Wnt3a wingless-related MMTV integration site 3A [<i>Mus musculus</i>]	2.25	0.0011	-1.75	0.0040
LAYN	layilin	2.34	0.0018	-1.48	0.0277
LOC38606		2.62	0.0011	-1.61	0.0141
AMPD3	adenosine monophosphate deaminase 3	2.64	0.0165	-2.26	0.0143
DNMT3L	similar to DNA cytosine-5 methyltransferase 3-like protein	2.92	0.0114	-1.84	0.0430
ESD	similar to esterase D/formylglutathione hydrolase	3.14	0.0011	-1.67	0.0155
LOC23488		3.19	0.0011	-1.67	0.0193
SLC11A1	solute carrier family 11 (proton-coupled divalent metal ion transporters), member 1	3.28	0.0008	-3.10	0.0017
SRXN1	sulfiredoxin 1 homolog (<i>S. cerevisiae</i>)	3.41	0.0002	-1.70	0.0046
BLVRB	biliverdin reductase B (flavin reductase (NADPH))	4.37	1.40E-05	-1.52	0.0056

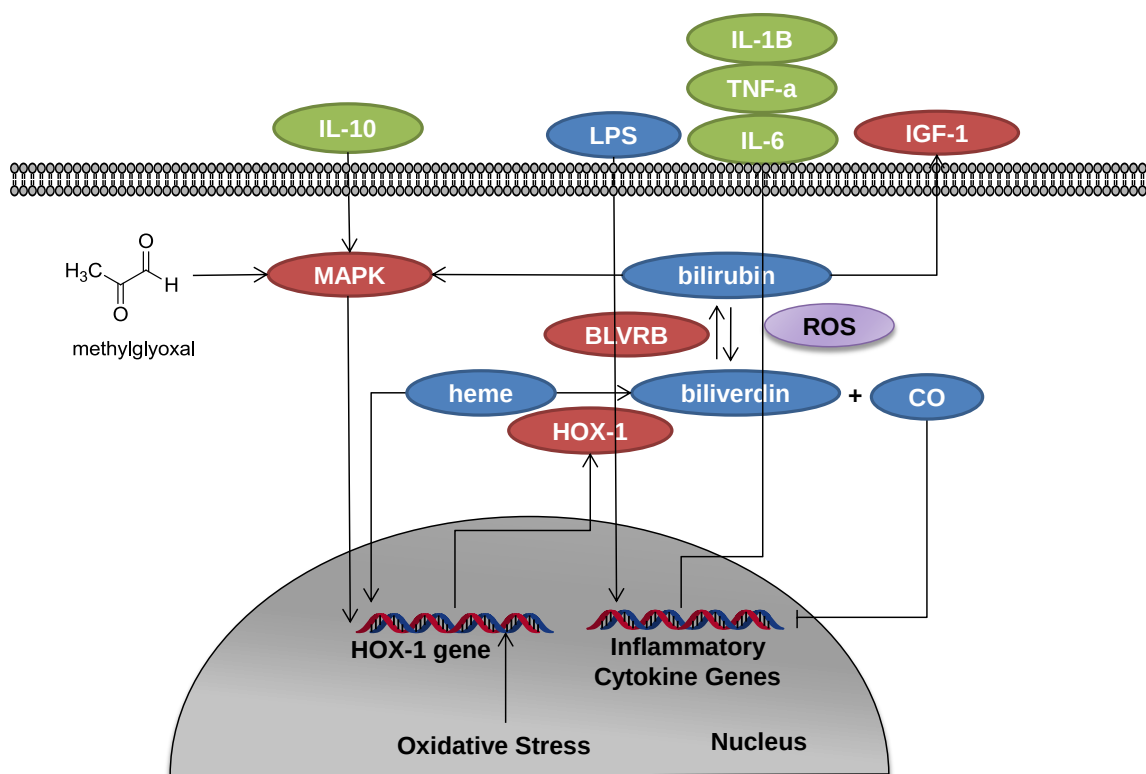


Figure 9.12. Network of pathways implicated in DPD immune suppression by the gene expression data.

due to DPD treatment are biliverdin reductase B (BLVRB) and glutamate-cysteine ligase (GCLM). Just upstream of BLVRB is heme oxygenase (HMOX1) which was also increased upon DPD interaction. Oxidative stress is known to increase HMOX1 expression, and this enzyme can then cleave heme to release an iron cation, biliverdin and carbon monoxide.²³⁹ The product carbon monoxide may then inhibit the expression of cytokines²⁴⁰ (Figure 9.12). The effects of anti-inflammatory cytokine IL-10, are also mediated by heme oxygenase,²⁴⁰ placing this protein at the crossroads of both oxidative stress and anti-inflammatory processes. Insulin-like growth factor is also increased in DPD treated cells (2.4 fold change, 0.0011 p-value), and this protein has been correlated with bilirubin levels^{241,242} as well as implicated to be part of an NF- κ B controlled anti-inflammatory pathway.²⁴³ The IL-10 anti-inflammatory pathway activates p38 MAPK, a cell stress response regulator, in order to modulate HMOX1 levels.²⁴⁰ This pathway can also be directly induced by reactive oxygen species, and further studies of methylglyoxal and

glyoxal showed p38 was the major contributor to IL-8 production in an intestinal cell line.²⁴⁴

9.7 Redox Activity of AI-2

Because DPD contains a very electron poor carbon center, we expected that the molecule could be redox active. There are several cases where AI-2 was implicated as a signal for a protective increase in oxidative stress response proteins²⁴⁵ and at least one where this response was directly attributed to the chemical properties of the molecule. *Mycobacterium avium* responds to DPD and hydrogen peroxide with the same stress phenotype.²⁴⁶ First efforts at characterizing DPD were completed with DPD in complex with iron, and in some species it is demonstrably involved in iron acquisition. We therefore hypothesized that either DPD itself caused the oxidative stress observed in the transcriptomics data set, or it bound enough catalytic metal centers to disrupt redox activities at those sites and cause oxidative stress.

Wild type *E. coli* would likely be under more oxidative stress than a *luxS*⁻ mutant, if DPD does function in this manner. Serial dilutions of oxidative stress inducing compounds phenazine, menadione and hydrogen peroxide were inoculated with either wild type or *luxS*⁻ *E. coli* cultures in a 96-well plate and observed the next day. A threshold concentration capable of allowing growth in the *luxS*⁻ strain but not the wild-type was sought. No threshold concentrations of this type were observed; however, even after narrowing the dilution window and decreasing the dilution factor between each sample well. Once a partially toxic concentration was identified, cultures of wild type, *luxS*⁻, and *IsrB*⁻ strains were inoculated as usual in Erlenmeyer flasks of LB spiked with the oxidative stressor at a sub-toxic concentration. Optical density was observed over time to see if one strain grew more easily under oxidative stress, late in the experiment the cultures were plated in order to count colony forming units (Figure 9.13). No differences in growth were observed by either method. We concluded that differences in oxidative stress were too small to observe by bulk behaviors.

To establish the redox potential of DPD, we initially attempted cyclic voltammetry (CV) experiments. There were several drawbacks to this approach. Namely, the experiments require a large sample volume at fairly high concentrations and previous the CV studies on α -diketones showed that the relevant redox reaction was irreversible.

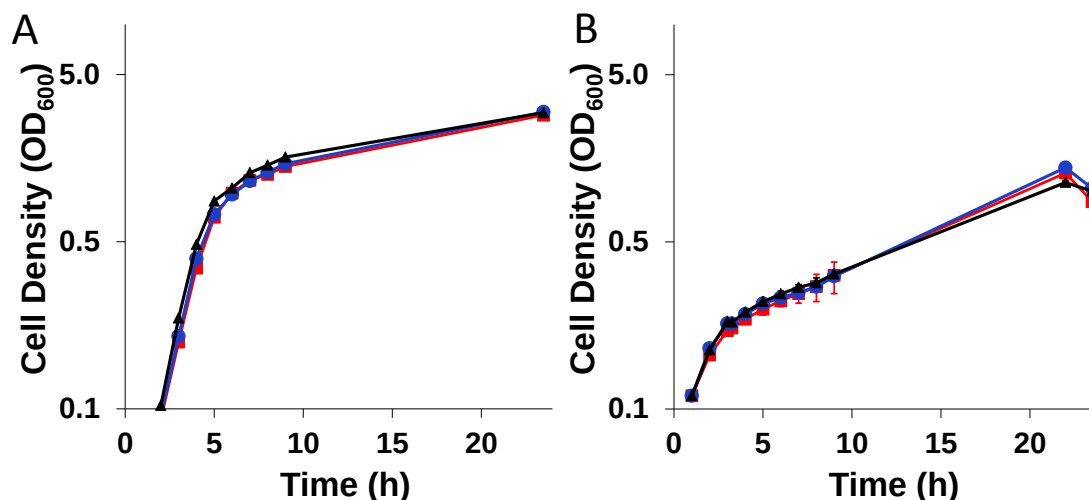


Figure 9.13. Growth of WT (■), *luxS*⁻ (●), and *lsrB*⁻ (▲) *E. coli* in LB containing sub toxic concentrations of A) menadione (300 μM) or B) phenazine (75 μM).

The molecule exists in both open and cyclic forms, which may also affect the measured potential. The limit of the redox potential measured is limited by the potential of the buffer (water) and dependent on the condition of electrode. This experiment was attempted in a phosphate-citrate buffer with KCl added as a charge carrier. The only redox chemistry observed was that of dissolved oxygen.

The relevant intracellular redox potentials are largely bracketed by the potential of the electron transport chain components. The electron source in this process is primarily NADPH, which shares the same redox potential with its less expensive relative NADH. NADH absorbs 340 nm light while NAD⁺ does not. It is therefore possible to check if DPD has a biologically relevant redox potential even if the exact potential cannot be determined by CV. Preliminary experiments have demonstrated that DPD can oxidize NADH.

9.8 Conclusions

Our studies in Chapter 8 have already shown that AI-2 quorum sensing signaling does not occur in all species. Here we have also demonstrated that LuxS dysfunction can be mitigated by recycling oxidized glutathione to cysteine and routing it back into the activated methyl cycle. This slight rerouting of metabolites is adequate to offset the extra

demand for homocysteine equivalents. *E. coli* is also known to expel much of its sulfur containing waste products rather than recycling them upon reaching stationary phase,²⁴⁷ demonstrating that *E. coli* generally has an abundance of these sulfur sources and has no use for them. Experiments thus far have also been unable to establish that Lsr transporter mediated importation of AI-2 serves a defensive purpose against competitive bacteria. These experiments indicate that none of the current hypotheses are sufficient to explain why so many species are capable of making AI-2.

Instead, we have found that AI-2 suppresses the innate immune response by disrupting cytokine signaling, thus implying that this molecule may help defend the bacteria during host colonization. The transcriptomics data show that the immune cells respond to AI-2 by increasing transcription of oxidative stress response proteins, decreasing the production of inflammatory response signals and increasing expression of cation transport and scavenging proteins. Future studies aim to determine the mechanism of the AI-2 host interaction. DPD itself may cause general oxidative stress, or its reaction with particular cellular components may cause this phenomenon. We intend to study whether direct induction of p38 MAPK by a DPD derived reactive oxygen species might cause the phenotype observed. The oxidation of NADH pools may lead to inhibition of enzymes including SIRT1,²⁴⁸ which deacetylates NF- κ B. Similarly, if DPD competitively complexes metals, other important enzymes may be inhibited. It is possible that DPD plays a general anti-inflammatory role, and this aspect will be pursued as well. Preliminary studies are already underway to characterize the redox potential and metal binding capabilities of AI-2.

9.9 Methods

General Methods. The liquid chromatography-tandem mass spectrometer (LC-MS/MS) used for these studies consisted of a Finnigan Surveyor Autosampler Plus, Surveyor MS Pump Plus, and TSQ Quantum Discovery Max triple quadrupole MS (Thermo Electron Corporation, Waltham, Massachusetts). All MS spectra were collected and analyzed using the Xcalibur MS software package (Thermo Electron Corporation). The following chemicals were commercially available as an ACS grade compound and used without further purification unless otherwise noted: ammonium bicarbonate (Analytical, Mallinckrodt), benzoic acid (Acros), peroxidase type 1 (MP Biomedicals), agarose (UltraPureTM, Invitrogen), sodium chloride (USP, VWR International). All other reagents

listed below were ordered from Fisher Scientific: water (HPLC), methanol (HPLC), acetonitrile (HPLC), ammonium acetate, ammonium hydroxide, tributylamine (99%), tris(hydroxymethyl)aminomethane (99+%), acetic acid, o-dianisidine, glycerol (99+%), glucose oxidase (>100U/mg), sodium phosphate monobasic, sodium phosphate dibasic, potassium sulfate (99+%), magnesium sulfate (sci ed), glucose, ammonium chloride (sci ed), agar (sci ed), tryptone, yeast extract, cyclohexanone, sulfuric acid

Bacterial Strains and Growth Conditions. *E. coli* wild type strain BW25113, *luxS*⁻ strain JW2662-1, and *IsrB*⁻ strain JW1509-1 were established as part of the Keio Collection¹⁹⁶ and purchased from the Coli Genetic Stock Center at Yale University. *E. coli* cultures were maintained in lysogeny broth (LB)¹⁸⁹ at 37 °C with aeration and 50 µg/mL kanamycin if applicable. To prepare the cells for minimal media experiments, an overnight culture of cells in LB was diluted 1:50 into minimal media and acclimatized for 36 h with an inoculation into fresh media at 24 h. All metabolism experiments were performed using a defined minimal media²⁴⁹ composed of 4.7 g KH₂PO₄, 13.5 g K₂HPO₄, 1 g K₂SO₄, 0.01MgSO₄ per liter, 0.01M NH₄Cl and optionally 1.5% w/v agarose was autoclaved, and supplemented with 0.4% w/v glucose. Isotopically labeled agarose plates for flux experiments were made up of the same defined minimal media with either the glucose replaced with (¹³C₆)glucose or ammonium chloride replaced with ¹⁵NH₄Cl.

Chromatographic Details for [DPD] Assay. For each sample, 10 µL was injected onto a Kinetex XB-C18 column (2.6 µm pore size, 100 Å particle size 100 × 2.1 mm Phenomenex). A flow rate of 150 µL/min was used, and the eluent was introduced directly into the MS for ion detection. The mobile phases were 0.1% acetic acid in HPLC grade water (solvent A) and HPLC grade acetonitrile (solvent B), and these were used to construct the following 7 min gradient elution profile: (t 0 min, 99% solvent A, 1% solvent B; t 0.5 min, 95% solvent A, 5% solvent B; t 3 min, 90% solvent A, 10% solvent B; t 5 min, 25% solvent A, 75% solvent B; t 6 min, 25% solvent A, 75% solvent B; t 6.5 min, 99% solvent A, 1% solvent B; t 7 min, 99% solvent A, 1% solvent B). All separations were performed with a column temperature of 25 °C.

Mass Spectrometry and Data Handling Details for the [DPD] Assay. MS analysis was performed as described in Chapter 2 section observing SRMs 381-202 and 382-

203. These SRMs were used to determine the relative signal arising from detection of DPD-M1CQ to that of (¹³C)DPD-M1CQ. After manual peak integration, the data were corrected for isotopic impurities and the following equation was used to calculate the [DPD]:

$$\frac{\text{Signal DPD-M1CQ}}{\text{Signal } (^{13}\text{C})\text{DPD-M1CQ}} \times [(^{13}\text{C})\text{DPD in solution A}] \times \frac{\text{volume solution A}}{\text{volume culture sampled}} = [\text{DPD}]$$

Determination of [DPD] in *E. coli* Cultures Grown in Minimal Media. *E. coli* wild type strain BW25113 was grown overnight, and then used to make a 2% (v/v) inoculum in fresh minimal media (25 mL) in 125 mL glass Erlenmeyer flasks. At the time intervals indicated in Figure 9.1, the cell density was determined by measuring the OD₆₀₀ for 0.7 mL aliquots of each culture, and separate 300 µL aliquots were taken for measurement of the [DPD]. These aliquots were added to a 1.5 mL microcentrifuge tube containing a solution of the internal standard (1-¹³C)DPD (341 µM in 10 µL H₂O). The resulting solution was mixed thoroughly and then centrifuged (13,200 rpm, 16,100 rcf, 1 min) to remove particulates. A portion of the resulting supernatant (260 µL) was then transferred to a 1.5 mL microcentrifuge tube containing a solution of the derivatizing agent M1CQ (14 mM in 26 µL H₂O). This solution was mixed thoroughly and after 45 min incubation at ambient temperature the samples were extracted 2 x 130 µL with ethyl acetate. The organic layer was saved in a 300 µL screw cap autosampler vial and stored at 4 °C until analysis.

***E. coli* growth on filters.** *E. coli* wild type strain BW25113 and *luxS*⁻ strain JW2662-1 were prepared for minimal media experiments as described in the *Bacterial Strains and Growth Conditions* section. These cultures were used to make a 2% (v/v) inoculum in fresh minimal media (25 mL) in 125 mL glass Erlenmeyer flasks. At the time intervals indicated in Figure 9.1, the cell density was determined by measuring the OD₆₀₀ for 0.7 mL aliquots of each culture, and separate 300 µL aliquots were taken for measurement of the [DPD]. These aliquots were added to a 1.5 mL microcentrifuge tube containing a solution of the internal standard (1-¹³C)DPD (341 µM in 10 µL H₂O). The resulting solution was mixed thoroughly and then centrifuged (13,200 rpm, 16,100 rcf, 1 min) to

remove particulates. A portion of the resulting supernatant (260 μ L) was then transferred to a 1.5 mL microcentrifuge tube containing a solution of the derivatizing agent M1CQ (14 mM in 26 μ L H₂O). This solution was mixed thoroughly and after 45 min incubation at ambient temperature the samples were extracted 2 x 130 μ L with ethyl acetate. The organic layer was saved in a 300 μ L screw cap autosampler vial and stored at 4 °C until analysis.

Metabolite Extraction. Samples were prepared by modification of methods of the Rabinowitz lab.²⁵ Prior to the experiment *E. coli* strains BW25113 and JW2662-1 were set back in minimal media overnight at 37 °C with aeration. The morning of the experiment, each strain was diluted 1:50 in fresh minimal media and incubated until the cultures reached approximately 0.1 OD₆₀₀. Aliquots (5 mL) of these cultures were then filtered onto Magna Nylon Filters (Millipore, Billerica MA) and the filters were placed on minimal agarose plates cell-side up. The plates were incubated at 37 °C for approximately 4 h or until the BW25113 reached an OD₆₀₀ of 0.4 as measured by sacrificing a plate by pipetting 5 mL of water over the surface to knock off any cells. For pool size measurements, metabolism was then stopped by transferring the filters quickly cell-side down to a 60 x 15 mm petri dish at -20 °C containing 1.3 mL of an extraction solvent (0.1 M formic acid in 1:2:2 water:methanol:acetonitrile) and internal standards (42.5 μ M tris(hydroxymethyl)aminomethane and 17 μ M benzoic acid). For flux measurements, filter cultures were switched to isotope labeled agarose plates and periodically transferred cell-side down to a petri dish at -20 °C containing extraction solvent (2, 5, 15, 30, and 60 min for ¹³C flux, and 2, 5, 10, 20, and 40 min for ¹⁵N flux). The samples were then allowed to extract for 20 min at -20 °C. The filters were then washed by pipetting the extraction solution over the surface for approximately 30 s. The solution was then centrifuged (5 min, 13.2 rpm, 16.1 rcf, 4°C) and 300 μ L of the supernatants was added to each of two autosampler vials containing 26 μ L of 15% ammonium bicarbonate. One vial was placed directly in the autosampler for analysis, and the other was frozen at -80°C for analysis in the opposite mode the following day.

Metabolomics Chromatographic Details. Metabolites were separated by modification of methods of the Rabinowitz lab.^{30,32} High performance liquid chromatography (HPLC)

was performed utilizing a quaternary pump to generate a gradient for the elution of compounds from the stationary phase. For all samples, 10 μ L was injected onto the column via an autosampler cooled to 4 °C. Two chromatographic runs were necessary for full analysis of the metabolome as separate detection is required for positively and negatively ionizing molecules. For positive mode analyses, a flow rate of 150 μ L / min was used. The stationary phase was aminopropyl functionalized particles (5 μ m pore size, 100 Å particle size) packed into a 250 $\times$ 2 mm column (Luna NH₂, Phenomenex, Torrance CA). The mobile phases were 95% 20mM ammonium acetate, 20mM ammonium hydroxide buffered at pH= 9.4 in HPLC grade water and 5% HPLC grade acetonitrile (solvent A) and HPLC grade acetonitrile (solvent B). These were used to construct the following 40 min gradient elution profile: t) 0 min, 15% solvent A, 85% solvent B; t) 15 min, 100% solvent A, 0% solvent B; t) 28 min, 100% solvent A, 0% solvent B; t) 30, 15% solvent A, 85% solvent B; t) 40, 15% solvent A, 85% solvent B. Separations were performed with the column at 10 °C. For negative mode analyses, a flow rate of 200 μ L / min was used. The stationary phase was a Synergi Hydro-RP C18 (4 μ m pore size, 80 Å particle size) packed into a 150 $\times$ 2 mm column (Phenomenex, Torrance CA). The mobile phases were 11mM tributylamine and 15mM acetic acid in 97% HPLC grade water and 3% HPLC grade methanol (solvent A) and HPLC grade methanol (solvent B). These were used to construct the following 50 min gradient elution profile: t) 0 min, 100% solvent A, 0% solvent B; t) 5 min, 100% solvent A, 0% solvent B; t) 10 min, 80% solvent A, 20% solvent B; t) 15, 80% solvent A, 20% solvent B; t) 30, 35% solvent A, 65% solvent B; t) 33, 5% solvent A, 95% solvent B; t) 37, 5% solvent A, 95% solvent B; t) 38, 100% solvent A, 0% solvent B; t) 50, 100% solvent A, 0% solvent B. Separations were performed with the column at 25 °C. The eluent was introduced directly into the MS for ion detection.

Metabolomics Mass Spectrometric Detection Parameters. Samples were introduced into the electrospray ionization (ESI) chamber of the triple quadrupole MS through a 0.1 mm internal diameter fused silica capillary after delivery by HPLC as described above. The spray voltage for the ESI source was set to 4500 V for detection in positive ion mode and 3000 V for detection in negative ion mode. Nitrogen was used as the sheath gas (40 psi), and the inlet capillary temperature was 290 °C. The argon used as the collision gas was set at 1.5 mTorr. Samples were analyzed using selected reaction

monitoring (SRM), and the scan time for each SRM was 0.05 s with a scan width of 1 *m/z*. See Table X for a list of SRMs. Full selected reaction monitoring (SRM) detection parameters for the majority of compounds have been previously reported by Rabinowitz and coworkers.

Metabolomics Data Handling. Xcalibur Quan Browser (Thermo Electron, Waltham MA) was used to visualize and manually integrate metabolite peaks over a set time window. The integration values were then entered in an excel spreadsheet and submitted to statistical analysis and visualization in Excel and Cluster.

Determination of [Glucose] A 150 μ L culture aliquot was added to a 1.5 mL microcentrifuge tube and then centrifuged (13,200 rpm, 16,100 rcf, 1 min) to remove cells and other particulates. A portion of the resulting supernatant (130 μ L) was then transferred to a 1.5 mL microcentrifuge tube, quickly frozen and stored at -80 °C for a maximum of 48 h until assayed. Samples (100 μ L) were analyzed at two concentrations, full concentration in the cell supernatants and at a 12:102 dilution, in a clear, flat-bottom, 96-well plate with an external calibration curve in each plate. Each well received a modified assay reagent (200 μ L), based on the method reported by Llyod and Whelan containing 0.30 M Tris, 0.36 M NaH₂PO₄ 2H₂O, 5.4 M glycerol, 1.0 mM o-dianisidine, 3 kU/ L peroxidase and 1 kU/L glucose oxidase type X in water. The plate was then incubated at 37 °C for 15 h. The absorbance at 500 nm was read the following day.

***E. coli* glucose uptake assay.** *E. coli* wild type strain BW25113 and *luxS*⁻ strain JW2662-1 were prepared for minimal media experiments as described in the *Bacterial Strains and Growth Conditions* section. These cultures were used to make a 2% (v/v) inoculum in fresh minimal media (25 mL) in 125 mL glass Erlenmeyer flasks. At the time intervals indicated, the cell density was determined by measuring the OD₆₀₀ for 0.7 mL aliquots of each culture, and separate 150 μ L aliquots were taken for measurement of the [glucose]. These aliquots were added to a 1.5 mL microcentrifuge tube and then centrifuged (13,200 rpm, 16,100 rcf, 1 min) to remove particulates. A portion of the resulting supernatant (130 μ L) was then transferred to a 1.5 mL quickly frozen and stored at -80 °C for a maximum of 48 h until assayed.

***E. coli* growth in nutrient limited media.** *E. coli* wild type strain BW25113 and *luxS*⁻ strain JW2662-1 were prepared for minimal media experiments as described in the *Bacterial Strains and Growth Conditions* section. These cultures were used to make a 2% (v/v) inoculum into each of eight flasks containing either minimal media, minimal media without added sulfate, minimal media without added glucose, or minimal media without added ammonium chloride (25 mL) in 125 mL glass Erlenmeyer flasks. At the time intervals indicated in the tables below, the cell density was determined by measuring the OD₆₀₀ for 0.7 mL aliquots of each culture.

***E. coli* growth on [DPD] as a food source.** A 4.8 mL DPD stock solution (4.7 mM) was deprotected with concentrated H₂SO₄ (4.8 µL) for 3 h in an Erlenmeyer flask. A control solution was made with 4.7 mM cyclohexanone and the same 0.1% v/v H₂SO₄, also in an Erlenmeyer flask. To each solution was added 2x minimal salts (4.8 mL). *E. coli* wild type strain BW25113 was grown overnight in LB. The cell density of this solution was then measured by optical density at 600 nm to be 2.49. The culture (5 mL) was washed with 2.5 mL 1x minimal salts twice by centrifugation and resuspension. The cell pellet was then resuspended in 1 mL so that a 0.5 mL inoculation into each of the Erlenmeyers yielded an optical density at 600 nm of 0.21. The flasks were incubated at 37 °C with aeration. At the time intervals indicated, the cell density was determined by measuring the OD₆₀₀ for 0.7 mL aliquots of each culture, and separate 300 µL aliquots were taken for measurement of the [DPD]. These aliquots were added to a 1.5 mL microcentrifuge tube containing a solution of the internal standard (1-¹³C)DPD (341 µM in 10 µL H₂O). The resulting solution was mixed thoroughly and then centrifuged (13,200 rpm, 16,100 rcf, 1 min) to remove particulates. A portion of the resulting supernatant (260 µL) was then transferred to a 1.5 mL microcentrifuge tube containing a solution of the derivatizing agent M1CQ (14 mM in 26 µL H₂O). This solution was mixed thoroughly and after 45 min incubation at ambient temperature the samples were extracted 2 x 130 µL with ethyl acetate. The organic layer was saved in a 300 µL screw cap autosampler vial and stored at 4 °C until analysis.

Determination of Cytokine Concentrations Released from RAW2 Cells. RAW264.7 cells were maintained in RPMI supplemented with 10 % BCS and 1 % L-glutamine at 37 °C and an atmosphere of 5 % carbon dioxide. Prior to experiments RAW264.7 cells were

split 1:3 into fresh media (RPMI 1640, 10% BCS, 1% Gln) and allowed to grow to confluence. The cells were then scraped from the surface to suspend them in the 12 mL media in the flask. This cell suspension was then centrifuged (1000 rpm, 5 min, 16 °C). The supernatant was decanted and the pellet was resuspended in 0.9 mL of fresh media. The cells were counted, and finally diluted to 5×10^5 cells/mL. This cell suspension (1 mL) was then added to each well in a 48-well plate and incubated for 6 h. During this time, serial dilutions of DPD were made by mixing 500 μ L of the 4.7 mM DPD stock solution with 500 μ L media, mixing thoroughly and transferring 500 μ L of this solution to a vial containing another 500 μ L media, and repeating once more. In the same fashion a series of serial dilutions were made with a 4.7 mM cyclohexanone solution (pH=1), to control for the other contents of the DPD stock solution. After 6 h, 42 μ L of either media, DPD solution (final concentration of 188, 94, 47, or 23 μ M), or cyclohexanone solution was added to each well, followed by 10 μ L of either media or LPS (10 ng/ μ L). The plates were stimulated over an 18 h period, and then supernatants were transferred into a microcentrifuge tube and centrifuged. These supernatants were transferred again and stored at -80 °C until analysis. Cytokines were measured by ELISA (IL-1 β , IL-6, TNF- α from BioLegend (San Diego, CA), and IFN- α kits from Pierce Protein Research (Thermo Fisher Scientific, Rockford, IL) and NO by the Griess assay (Promega, Madison, WI) according to the manufacturer's instructions.

Determination of Gene Expression Levels in RAW2 Cells. RNA was isolated from the cell pellets from the cytokine experiments above using an RNeasy Kit (Qiagen, Germantown, MD) according to the manufacturer's instructions. The RNA was tested for quality, then shipped to McGill University and Genome Quebec Innovation Centre for microarray experiments. Statistical analysis was completed on the resulting data, and trends in those probes that were significantly different in gene expression level were analyzed using the DAVID gene ontology program.

Determination of [DPD] in Murine GI Segments. All animal studies were approved by the University of Tennessee Institutional Animal Care and Use Committee. A 4.7 mM DPD stock solution of (1-¹³C)DPD was diluted 10 μ L into 460 μ L of H₂O, followed by another dilution of 20 μ L into 180 μ L, to make a 10 μ M working stock solution of (1-

¹³C)DPD internal standard. Six C57BL/6J male mice fed continuously on a low fat diet (10%) were euthanized by carbon dioxide asphyxiation. The stomach, ~ 3 cm of small intestine starting from the distal sphincter of the stomach, cecum, and the descending colon were harvested. The contents of each were removed and collected into each of four preweighed eppendorf tubes containing derivatizing solution (5 mg/mL in 300 μ L H₂O). As soon as possible, 3 μ L of the working stock solution of (1-¹³C)DPD was added to the suspended solution of tag and contents and mixed thoroughly. Solutions sat at RT for at least 1 hr, and during this time were weighed to determine the mass of the contents added. The tubes then centrifuged (13,200 rpm, 16,100 rcf, 5 min), chilled at 4 °C for at least 30 min to facilitate any precipitation, and centrifuged again. The supernatants were stored at 4 °C until mass spectrometric analysis.

***E. coli* DPD production in response to epinephrine.** *E. coli* wild type strain BW25113, *luxS*⁻ strain JW2662-1, and *IsrB*⁻ strain JW1509-1 were grown overnight, and then used to make a 2% (v/v) inoculum in fresh LB (25 mL) or LB containing epinephrine (50 μ M) in 125 mL Erlenmeyer flasks. At the time intervals indicated, the cell density was determined by measuring the OD₆₀₀ for 0.7 mL aliquots of each culture, and separate 300 μ L aliquots were taken for measurement of the [DPD]. These aliquots were added to a 1.5 mL microcentrifuge tube containing a solution of the internal standard (1-¹³C)DPD (341 μ M in 10 μ L H₂O). The resulting solution was mixed thoroughly and then centrifuged (13,200 rpm, 16,100 rcf, 1 min) to remove particulates. A portion of the resulting supernatant (260 μ L) was then transferred to a 1.5 mL microcentrifuge tube containing a solution of the derivatizing agent M1CQ (14 mM in 26 μ L H₂O). This solution was mixed thoroughly and after 45 min incubation at ambient temperature the samples were extracted 2 x 130 μ L with ethyl acetate. The organic layer was saved in a 300 μ L screw cap autosampler vial and stored at 4 °C until analysis.

***E. coli* growth in the presence of oxidative stress.** *E. coli* wild type strain BW25113, *luxS*⁻ strain JW2662-1, and *IsrB*⁻ strain JW1509-1 were grown overnight, and then used to make a 2% (v/v) inoculum in fresh LB (25 mL), and LB containing either phenazine (75 μ M) or menadione (300 μ M), in 125 mL Erlenmeyer flasks. At the time intervals indicated, the cell density was determined by measuring the OD₆₀₀ for 0.7 mL aliquots of

each culture. At 9 h growth 10 μ L of each culture was diluted serially and spread on LB plates to determine colony forming units.

Cyclic Voltammetry. A freshly polished glassy carbon working electrode, was used in conjunction with a platinum auxillary electrode, and Ag/AgCl reference electrode to measure the current of each of four solutions from 0 to -1.4 V. Solutions were made up in a McIlvaine buffer of sodium phosphate (0.174 M), citric acid (0.013 M) and potassium chloride (40.4 g/L). This buffer was diluted 2x with either water, 2 mM 2,3-butanedione, 2 mM acidic cyclohexanone, or 2 mM DPD to make each of the four solutions tested.

CHAPTER X

Conclusions and Future Directions

As the field of metabolomics continues to evolve, it is clear that metabolomics is becoming a very promising tool capable of impacting a diversity of fields. While this technique has received much attention for its potential as a diagnostic screening tool, we would like to emphasize its importance for basic science studies as well. There is still much to learn about metabolism. Even in *E. coli* only slightly more than half of the genes have been assigned a function. Some of these will be enzymes, and discovery metabolite profiling is a powerful means of characterizing these genes. The one study we have completed herein has challenged the canonical pentose phosphate pathway, and defined a new metabolic pathway.

We have participated in this evolution by developing a flux method capable of handling liquid cultures and species in coculture. The composition of a community has bearing on the metabolism of the individual and the metabolism of the community, and we believe this coculture method will be of importance to studying this phenomenon in many systems outside the scope of our current study. The species investigated here is the most abundant photosynthetic organism on earth, and its function as a carbon dioxide scavenger and primary producer will be important for environmental protection strategies. Building a broader understanding of carbon flux through the oceans will yield a better model of the global food web.

The DPD detection method described here opens the door for quantitative experiments in the field of quorum sensing. Up to this point, there has been little known about the biologically relevant concentrations of AI-2, and we have bench marked concentrations for the oral cavity, the gastrointestinal tract, and sinking particles in the ocean (not discussed). The method also enables the direct quantitation of DPD, not a subsequent phenotype, thereby enabling experiments following rates of production, consumption, and biosynthesis. These types of studies enabled us to develop a quantitative definition of quorum sensing and propose several other types of information carried by these signals. Importantly, there is now an objective means to rule that some species use AI-2 for quorum sensing, while others like *E. coli* do not. As a result, quorum signaling is still considered an important function of this molecule, but the universal reach of this system has been limited. Other hypotheses such as the metabolic and

quorum quenching arguments were found to be inadequate to explain the broad spectrum of bacteria capable of making DPD.

Instead, we propose that AI-2 is a defense mechanism capable of suppressing the host immune system by disrupting cytokine signaling. Transcriptomics analyses implicated immune response and redox chemistry as the important effects of DPD treatment. These two effects are very much tied together, and it is as yet unclear whether general oxidative stress, like radiation, is causing a decrease in cytokine production, or if DPD reacts in a more targeted fashion. Further work is yet needed to characterize the mechanism of action of DPD, and studies are underway.

This work calls into question the potential for AI-2 signaling as an anti-infective target. However, building a better understanding of infectious processes, especially of such a potentially wide-spread virulence factor, may lead to new strategies to fight disease. For example, identification of the AHL mechanism of immune modulation, PPARs, led to the trial of a PPAR agonist in a cystic fibrosis model, where it was successful in attenuating inflammation.²⁵⁰ We have no doubt that S-4,5-dihydroxy-2,3-pentanedione will continue to be an interesting and important molecule.

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APPENDIX

Table A2.1 SRMs in Negative Mode Method

Polarity	compound	KEGG ID	Parent Formula	Product Formula	Parent Mass	Product Mass	Collision Energy	Expected RT	0-17	17-28	28-35	35-50	Notes
-	Glyoxylate	C00048	C ₂ H ₃ O ₃ ⁻		73	45	20	12.7	x				
-	Glycolate	C00160	C ₂ H ₃ O ₃ ⁻		75	45	11	12.5	x				
-	Pyruvate	C00022	C ₃ H ₃ O ₃ ⁻	C ₂ H ₃ O ⁻	87	43	12	15	x	x			
-	<u>Lactate</u>	C00186 C00256	C ₃ H ₅ O ₃ ⁻	C ₂ H ₃ O ⁻	89	43	14	12.3	x				big peak is Glucose-related
-	2(3)-Oxobutanoate (aka acetoacetate)	C00109 C00164	C ₄ H ₅ O ₃ ⁻	C ₃ H ₅ O ⁻	101	57	11	14	x	x			
-	Glycerate	C00258	C ₃ H ₅ O ₄ ⁻	C ₂ H ₃ O ₃ ⁻	105	75	15	12	x				
-	Uracil	C00106	C ₄ H ₃ N ₂ O ₂ ⁻	CNO ⁻	111	42	20	3	x				
-	Fumarate, Maleate, and iso-Ketovaleate	C00122 C01384 C00141	C ₄ H ₃ O ₄ ⁻	C ₃ H ₃ O ₂ ⁻	115	71	11	24.6		x	x		the later big peak is from fumarate
-	Guanidoacetate	C00581	C ₃ H ₆ N ₃ O ₂ ⁻		116	74	25	4	x				
-	<u>Succinate</u> and Methylmalonate	C00042 C02170	C ₄ H ₅ O ₄ ⁻	C ₃ H ₅ O ₂ ⁻	117	73	10	17.4	x	x			FWHM 0.2 for Q1 and Q3
-	IS-Succinate				119	74	10						
-	Benzoic Acid (IS)				121	77	13	27.5		x	x		
-	Nicotinate	C00253	C ₆ H ₄ NO ₂ ⁻	C ₅ H ₄ N ⁻	122	78	14	22.5		x	x		
-	Taurine	C00245	C ₂ H ₆ NO ₃ ⁻ S ⁻	SO ₃ ⁻	124	80	16	2.5	x				
-	Citraconate	C02226	C ₅ H ₅ O ₄ ⁻		129	85	10	29	x	x	x		
-	N-Acetyl-L-alanine				130	88	25	14.2	x				
-	Oxaloacetate	C00036	C ₄ H ₃ O ₅ ⁻	C ₃ H ₃ O ₃ ⁻	131	87	12	25.5	x				
-	4,5-dihydroxy-2,3-pentanedione (DPD)?		C ₅ H ₇ O ₄ ⁻	C ₄ H ₅ O ₃ ⁻	131.01	101	10	2.5	x				not sure about the peak, too big for DPD
-	<u>Malate</u>	C00711	C ₄ H ₅ O ₅ ⁻	C ₄ H ₃ O ₄ ⁻	133	115	12	19.8		x			
-	133-43 (deoxyribose?)				133.01	43	15	20.7		x			
-	Hypoxanthine	C00262	C ₅ H ₃ N ₄ O ⁻	C ₄ H ₂ N ₃ ⁻	135	92	16	3	x				
-	Aminobenzoate (pABA and oABA)	C00108 C00568	C ₇ H ₆ NO ₂ ⁻	C ₆ H ₆ N ⁻	136	92	16	24.9	x	x	x	x	
-	4-Hydroxybenzoate	C00156	C ₇ H ₅ O ₃ ⁻	C ₆ H ₅ O ⁻	137	93	21	28.6		x	x		
-	Acetyl phosphate	C00227	C ₂ H ₄ O ₅ P ⁻	PO ₃ ⁻	139	79	22	12.5	x				
-	Carbamoyl phosphate	C00169	CH ₃ NO ₅ P ⁻	PO ₃ ⁻	140	79	22	25		x			
-	Adipate and 2-Methylglutarate	C06104	C ₆ H ₉ O ₄ ⁻		145	83	25	26.5		x	x		
-	2-Oxoglutarate and Phenylpropiolate	C00026	C ₅ H ₅ O ₅ ⁻	C ₄ H ₅ O ₃ ⁻	145	101	11	21	x	x	x	x	

Table A2.1 Continued

Polarity	compound	KEGG ID	Parent Formula	Product Formula	Parent Mass	Product Mass	Collision Energy	Expected RT	0-17	17-28	28-35	35-50	Notes
-	2-Hydroxy-2-methylbutanedioate	C02612	C ₅ H ₇ O ₅ ⁻		147	85	25	26		x			
-	4-Methylthio-2-oxobutanoate	C01180	C ₅ H ₇ O ₃ S ⁻		147	99	11	30.1			x		
-	Xanthine	C00385	C ₅ H ₃ N ₄ O ₂ ⁻	C ₄ H ₂ N ₃ O	151	108	21	9	x				
-	2,3-Dihydroxybenzoate	C00196	C ₇ H ₅ O ₄ ⁻	C ₆ H ₅ O ₂ ⁻	153	109	17	30			x		
-	Orotate	C00295	C ₅ H ₃ N ₂ O ₄ ⁻	C ₄ H ₃ N ₂ O ₂	155	111	13	15.6	x	x			
-	Dihydroorotate	C00337	C ₅ H ₅ N ₂ O ₄ ⁻	C ₄ H ₅ N ₂ O ₂	157	113	12	13	x				
-	Allantoin	C01551	C ₄ H ₅ N ₄ O ₃ ⁻	C ₃ H ₄ N ₃ O ₂	157.01	114	15	2.3	x				
-	Aminoadipate and Indole-3-carboxylate	C00956 C19837			160	116	25	6	x		x		
-	Phenylpyruvate	C00166	C ₉ H ₇ O ₃ ⁻	C ₇ H ₇ ⁻	163	91	11	18.6		x			
-	Phenyllactate	C05607			165	103	25	34			x	x	
-	Atrolactate				165	119	25	33.5			x		
-	2,3-Pyridine dicarboxylate	C03722	C ₇ H ₄ NO ₄ ⁻	C ₆ H ₄ NO ₂	166	122	12	33			x		
-	Phosphoenolpyruvate	C00074	C ₃ H ₄ O ₆ P ⁻	PO ₃ ⁻	167	79	20	24.7		x			
-	Dihydroxyacetone-phosphate (DHAP)	C00111	C ₃ H ₆ O ₆ P ⁻	PO ₃ ⁻	169	79	38	12.5	x				
-	D-glyceraldehyde-3-phosphate	C00118	C ₃ H ₆ O ₆ P ⁻	H ₂ PO ₄ ⁻	169.01	97	12	12.5	x				it comes before DHAP
-	Glycerol-3-phosphate	C00093	C ₃ H ₈ O ₆ P ⁻	PO ₃ ⁻	171	79	13	13	x	x			
-	Shikimate	C00493	C ₇ H ₉ O ₅ ⁻	C ₆ H ₅ O ⁻	173	93	18	22		x			
-	pyrophosphate	C00013			173	159	10	31			x		
-	Aconitate (cis and trans)	C00417 C02341			173.01	85	15	25.8			x		
-	Ascorbate	C00072			175	87	25	14.9			x		
-	2-Isopropylmalate	C02504			175	115	25	29.7	x	x			
-	Allantoate and Carbamoyl-aspartate	C00499	C ₄ H ₇ N ₄ O ₄ ⁻	C ₃ H ₆ N ₃ O ₃	175	132	12	8	x				
-	Glucono-δ-lactone	C03107 C00198	C ₆ H ₉ O ₆ ⁻	C ₅ H ₅ O ₄ ⁻	177	129	11	12	x				
-	<i>myo</i> -inositol	C00137	C ₆ H ₁₁ O ₆ ⁻	C ₆ H ₉ O ₅ ⁻	179	161	15	1.5	x				contamination from glucose
-	4-Hydroxyphenylpyruvate	C01179	C ₉ H ₇ O ₄ ⁻	C ₇ H ₇ O ⁻	179.01	107	11	25.1		x	x		
-	Homocysteic acid	C16511	C ₄ H ₈ NO ₅ S ⁻	SO ₃ ⁻	182	80	21	10.6	x				
-	4-Pyridoxate	C00847			182	138	25	32.5			x		
-	3-Phosphoglycerate	C00597	C ₃ H ₆ O ₇ P ⁻	H ₂ PO ₄ ⁻	185	97	15	25.1		x	x		

Table A2.1 Continued

Polarity	compound	KEGG ID	Parent Formula	Product Formula	Parent Mass	Product Mass	Collision Energy	Expected RT	0-17	17-28	28-35	35-50	Notes
-	Indoleacrylate				186	142	25	35			x	x	
-	Kynurenate	C01717			188	144	19	32			x		
-	<u>Citrate</u> and Isocitrate	C00158 C00311	C ₆ H ₇ O ₇ ⁻	C ₅ H ₃ O ₃ ⁻	191	111	13	23.7		x	x		
-	Isocitrate	C00311	C ₆ H ₇ O ₇ ⁻	C ₄ O ₄ H ₅ ⁻	191.01	117	17	23.7		x	x		
-	<u>Citrate</u>	C00158	C ₆ H ₇ O ₇ ⁻	C ₃ O ₃ H ₃ ⁻	191.02	87	20	23.7		x	x		
-	2-Keto-D-gluconate	C06473	C ₆ H ₉ O ₇ ⁻	C ₄ H ₇ O ₃ ⁻	193	103	12	12.5	x				
-	Gluconate	C00257	C ₆ H ₁₁ O ₇ ⁻	C ₅ H ₅ O ₄ ⁻	195	129	15	11.4	x				
-	Erythrose-4-phosphate	C00279	C ₄ H ₈ O ₇ P ⁻	H ₂ PO ₄ ⁻	199	97	17	15.4	x				
-	Xanthurenate	C02470			204	160	25	32				x	
-	Glucarate	C00767	C ₆ H ₉ O ₈ ⁻	C ₄ H ₅ O ₂ ⁻	209	85	15	29		x	x		
-	2-Deoxyribose 1-phosphate		C ₅ H ₁₀ O ₇ P ⁻	PO ₃ ⁻	213	79	33	12.7	x				
-	Pantothenate	C00864	C ₉ H ₁₆ NO ₅ ⁻	C ₇ H ₁₆ NO ₂ ⁻	218	146	19	19.6	x	x			
-	Prephenate	C00254	C ₁₀ H ₉ O ₆ ⁻	C ₆ H ₃ O ⁻	225	91	15	31				x	
-	Deoxyuridine	C00526	C ₉ H ₁₁ N ₂ O ₅ ⁻	C ₈ H ₁₀ NO ₄ ⁻	227	184	12	3.6	x				
-	Bisphenol A (BPA)				227.01	133	24	34.5			x	x	
-	Bisphenol A (BPA)				227.02	212	22	34.5			x	x	
-	Pentose-phosphate (<u>ribose-5P</u>)	C00117 C00199 C00231	C ₅ H ₁₀ O ₈ P ⁻	PO ₃ ⁻	229	79	40	12.2	x				
-	240-179 (5-methyl deoxycytidine?)				240	179	10	1.9	x				
-	<u>Thymidine</u>	C00214	C ₁₀ H ₁₃ N ₂ O ₅ ⁻	C ₅ H ₅ N ₂ O ₂ ⁻	241	125	15	12	x				
-	<u>Uridine</u>	C00299	C ₉ H ₁₁ N ₂ O ₆ ⁻	C ₈ H ₁₀ NO ₅ ⁻	243	200	19	3	x				
-	Deoxyinosine	C05512	C ₁₀ H ₁₁ N ₄ O ₄ ⁻	C ₅ H ₃ N ₄ O ⁻	251	135	19	10	x				
-	Shikimate-3-phosphate	C03175			253	97	17	12.6	x				
-	Thymidine				253	132	15						
-	253-152				253.01	152	10	20		x			
-	Shikimate-3-phosphate	C03175	C ₇ H ₁₀ O ₈ P ⁻	H ₂ PO ₄ ⁻	253.1	97	17						no standard
-	Palmitate	C00249			255	237	25	37				x	
-	D-glucono-δ-lactone-6-phosphate	C01236	C ₆ H ₁₀ O ₉ P ⁻	H ₂ PO ₄ ⁻	257	97	20	27.5		x	x		no-standard
-	D-hexose-phosphate (<u>fructose-6P</u>)	C02672	C ₆ H ₁₂ O ₉ P ⁻	PO ₃ ⁻	259	79	40	12.1	x				

Table A2.1 Continued

Polarity	compound	KEGG ID	Parent Formula	Product Formula	Parent Mass	Product Mass	Collision Energy	Expected RT	0-17	17-28	28-35	35-50	Notes
-	Glucose-1-phosphate	C00103			259.01	241	14	17.8	x				G1P is 0.5 min later than G6P
-	<u>Glucose-6-phosphate</u>	C00092			259.02	199	13	12.1	x			x	G6P is 0.5 min earlier than G1P
-	<u>Fructose-6-phosphate</u>	C00085			259.03	169	14	12.1	x				
-	259-110 (a hexose phosphate?)				259.04	110	22	5	x				
-	Glycerate-diphosphate (1,3 and 2,3)	C00236	$C_3H_7O_{10}P_2^-$	PO_3^-	265	79	35	37			x	x	only 2,3-DP G standard
-	Glycerate-2,3-diphosphate				265.01	167	12	34			x	x	
-	S-Ribosyl-Homocysteine (SRH)	C03539			266	134	18	2.3	x				
-	<u>Inosine</u>	C00294	$C_{10}H_{11}N_4O_5$	$C_5H_3N_4O$	267	135	25	10	x				
-	D-Gluconate-6-phosphate	C00345	$C_6H_{12}O_{10}P^-$	$H_2PO_4^-$	275	97	11	29		x	x		
-	Xanthosine	C01762	$C_{10}H_{11}N_4O_6$	$C_5H_3N_4O_6$	283	151	22	14	x				
-	Stearate	C01530			283.01	265	25	38				x	
-	Sedoheptulose-7-phosphate	C05382	$C_7H_{14}O_{10}P^-$	PO_3^-	289	97	25	25		x			C12-method only
-	298-136 (hydroxyguanosine, sphingosine, or palmitoylethanolamide?)				298	136	27	5	x				
-	N-acetyl-glucosamine-1-phosphate	C04256	$C_8H_{15}NO_9P^-$	PO_3^-	300	79	32	13	x				
-	306-143 (dCMP or Glutathione?)				306	143	17	27.3		x	x		
-	dUMP	C00365	$C_9H_{12}N_2O_8P^-$	$C_5H_8O_6P$	307	195	16	20		x			new RT
-	Ribulose-1,5-bisphosphate	C01182			309	211	14	25.2		x			
-	Geranylpyrophosphate	C00353	$C_{10}H_{19}O_7P_2^-$	PO_3^-	313	79	18	42				x	
-	TMP	C00364			321	195	20	18.1		x			
-	cyclic-AMP	C00575	$C_{10}H_{11}N_5O_6P^-$	$C_5H_4N_5^-$	328	134.00	31	30			x		
-	<u>Fructose-1,6-bisphosphate</u>	C00354	$C_6H_{13}O_{12}P_2^-$	$H_2PO_4^-$	339	97	28	25		x	x	x	
-	339-110				339.01	110	30	20		x			
-	Dihexose (trehalose)	C01083	$C_{12}H_{21}O_{11}^-$	$C_6H_{11}O_6^-$	341	179	18	1.7	x				
-	Orotidine-phosphate	C01103	$C_{10}H_{12}N_2O_{11}P^-$	$C_9H_{12}N_2O_9P^-$	367	323	16	36			x	x	
-	Sedoheptulose-1,7-bisphosphate	C00447			369.01	97	35		x	x	x	x	

(b)

Table A2.1 Continued

Polarity	compound	KEGG ID	Parent Formula	Product Formula	Parent Mass	Product Mass	Collision Energy	Expected RT	0-17	17-28	28-35	35-50	Notes
-	Sedoheptulose-1,7-bisphosphate (a)	C00447			369	271	15		x	x	x	x	
-	trans, trans-farnesyl diphosphate	C00448	$C_{15}H_{27}O_7P_2^-$	PO_3^-	381	79	21	42				x	
-	S-adenosyl-L-homocysteine (SAH)	C00021			383	134	29	13	x				
-	dCDP	C00705	$C_9H_{14}N_3O_{10}P_2^-$	$HO_6P_2^-$	386	159	25	33.1			x		
-	5-Phospho-D-ribose-1-diphosphate (PRPP)	C00119	$C_5H_{12}O_{14}P_3^-$	$C_5H_9O_{10}P_2^-$	389	291	18	35.7			x	x	
-	Deoxycholate	C11171			391	345	40	42.2				x	
-	TDP	C00363			401	159	25	30.4			x		
-	CDP	C00112			402	384	20	29.5			x		CDP is 1min earlier than UDP
-	UDP	C00015			403	159	26	27.5	x	x			UDP is about 1min after CDP
-	Cholate	C00695			407	345	40	42				x	
-	Trehalose-6-Phosphate	C00689	$C_{12}H_{22}O_{14}P^-$	PO_3^-	421	79	34	12.5	x				C12-method only; called "AICAR" in EcoCyc
-	Thiamine pyrophosphate	C00068			423	302	25	18	x				
-	Adenosine 5'-phosphosulfate (APS)	C00224	$C_{10}H_{13}N_5O_{10}PS^-$	$C_{10}H_{13}N_5O_7P^-$	426	346	20	32			x		big peak is from ADP(RT32)
-	dGDP(SRM also for ADP)	C00361	$C_{10}H_{14}N_5O_{10}P_2^-$	$HO_6P_2^-$	426.01	159	25	31.7			x		big peak is from ADP(RT32)
-	IDP	C00104			427	159	25	32			x		IDP comes after ADP
-	GDP	C00035			442	159	19	25.6			x	x	
-	FMN	C00061	$C_{17}H_{20}N_4O_9P^-$	$C_5H_{10}O_7P^-$	455	213	19	27.8			x	x	
-	Cholesteryl sulfate	C18043			465	97	40	44				x	
-	dCTP	C00458	$C_9H_{15}N_3O_{13}P_3^-$	$HO_6P_2^-$	466	159	28	34	x	x			
-	dUTP	C00460	$C_9H_{14}N_2O_{14}P_3^-$	$HO_6P_2^-$	467	159	25	27.5	x	x			
-	477-417				477	417	10	36.3				x	
-	TTP	C00459			481	159	31	31.1			x	x	check IS for correct RT
-	CTP	C00063			482	159	32						
-	CTP	C00063			482	384	22	34			x	x	check IS for correct RT

- UTP C00075 483 159 33 34 x x x check IS for correct RT

Table A2.1 Continued

Polarity	compound	KEGG ID	Parent Formula	Product Formula	Parent Mass	Product Mass	Collision Energy	Expected RT	0-17	17-28	28-35	35-50	Notes
-	483-385				483.01	385	21	28.2	x	x			
-	dATP	C00131	C ₁₀ H ₁₅ N ₅ O ₁₂ P ₃	HO ₆ P ₂ ⁻	490	159	27	39				x	
-	TTP	C00459			493	159	31						
-	IS CTP/UTP				494	159	32						CTP earlier than UTP
-	Taurodeoxycholate	C05463			498	124	25	41.5				x	
-	ATP and dGTP	C00002			506	159	28	29	x	x	x		same SRM as dGTP; big peak is from ATP(RT35) check IS for correct RT
-	GTP	C00044			522	424	23	27.2	x	x	x		
-	UDP-D-glucose	C00029	C ₁₅ H ₂₁ N ₂ O ₁₈ P ₂ ⁻	C ₉ H ₁₂ N ₂ O ₉ P ⁻	565	323	23	24.7	x				
-	UDP-D-glucuronate and UDP-galacturonate	C00167 C00617	C ₁₅ H ₂₁ N ₂ O ₁₈ P ₂ ⁻	C ₉ H ₁₃ N ₂ O ₁₂ P ₂ ⁻	579	403	24	34	x	x	x		
-	ADP-D-glucose	C00498	C ₁₆ H ₂₄ N ₅ O ₁₅ P ₂ ⁻	C ₁₀ H ₁₃ N ₅ O ₇ P ⁻	588	346	22	26	x				
-	Guanosine tetraphosphate (ppGpp)	C01228			602	504	22	36			x	x	
-	UDP-N-acetylglucosamine	C00043			606	385	26	24.8	x				
-	611-306 (Ox. Glutathione or DG?)				611	306	28	23.2	x				
-	662-540 (Ceramide or PE)				662	540	19	15	x				
-	664-408 (NADH or DG?)				664	408	31	40				x	
-	Dephospho-CoA	C00882	C ₂₁ H ₃₄ N ₇ O ₁₃ P ₂ S ⁻		686.2	339	35	38				x	
-	Cyclic-diGMP	C16463			689	344	32	26.6	x	x			
-	709-354				709	354	32		x	x			
-	742-620 (PC or PE?)				742	620	18	25	x				
-	744-408 (NADPH, PC or PE?)				744	408	34	35			x	x	
-	CoA	C00010	C ₂₁ H ₃₅ N ₇ O ₁₆ P ₃ S ⁻		766	408	40	35				x	negative is better than positive
-	acetyl-CoA	C00024	C ₂₃ H ₃₇ N ₇ O ₁₇ P ₃ S ⁻		808	408	31	31.1				x	negative is better than positive
-	Propionyl-CoA	C00100	C ₂₄ H ₃₉ N ₇ O ₁₇ P ₃ S ⁻	ADP - H ₂ O	822	408	31	38				x	
-	Acetoacetyl-CoA	C00332	C ₂₅ H ₃₉ N ₇ O ₁₈ P ₃ S ⁻	- C ₄ H ₄ O ₂	850	766	30	36.6				x	
-	Malonyl-CoA	C00083	C ₂₄ H ₃₇ N ₇ O ₁₉ P ₃ S ⁻	-CO ₂	852	808	24	36.5				x	

-	Succinyl-CoA and Methylmalonyl-CoA	C00091	$C_{25}H_{39}N_7O_{19}P_3S^-$	$C_{24}H_{39}N_7O_{17}P_3S^-$	866	822	20	37		x	they cannot be separated
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Table A2.1 Continued

Polarity	compound	KEGG ID	Parent Formula	Product Formula	Parent Mass	Product Mass	Collision Energy	Expected RT	0-17	17-28	28-35	35-50	Notes
-	3-hydroxy-3-methylglutaryl-CoA	C00356	$C_{27}H_{43}N_7O_{20}P_3S^-$	$C_{10}H_{12}N_5O_9P_2^-$	910	408	43	37				x	

Table A2.2 SRMs in Positive Mode Method

Polarity	compound	KEGG ID	Parent Formula	Product Formula	Parent Mass	Product Mass	Collision Energy	Expected RT	0-11	11-15	15-19	19-40	Notes
+	<u>Urea</u>	C00086	CH ₅ N ₂ O ⁺		61	44	23	9.2	x				
+	<u>Imidazole</u>	C01589	C ₃ H ₅ N ₂ ⁺		69	42	21	6.9	x				
+	<u>Glycine</u>	C00037	C ₂ H ₆ NO ₂ ⁺	CH ₄ N ⁺	76	30	16	14.4		x	x		
+	<u>Alanine</u> and <u>Sarcosine</u>	C00041 C00213	C ₃ H ₈ NO ₂ ⁺	C ₂ H ₆ N ⁺	90	44	11	13.6		x	x		Alanine interference ! big peak will be Alanine
+	93-57				93	57	10		x	x	x	x	
+	Betaine Aldehyde	C00576	C ₅ H ₁₂ NO ⁺	C ₃ H ₆ N ⁺ ?	102	58	25	14.5		x	x		
+	Dimethylglycine	C01026	C ₄ H ₁₀ NO ₂ ⁺	C ₃ H ₆ N ⁺ ?	104	58	25	8.3	x	x			
+	Choline	C00114	C ₅ H ₁₄ NO ⁺	C ₃ H ₁₀ N ⁺	104.01	60	19	13.8		x	x		
+	GABA	C00334	C ₄ H ₁₀ NO ₂ ⁺		104.02	69	20	13.5		x			
+	<u>Serine</u>	C00065	C ₃ H ₈ NO ₃ ⁺	C ₂ H ₆ NO ⁺	106	60	13	14.7		x	x		
+	<u>Cytosine</u>	C00380	C ₄ H ₆ N ₃ O ⁺	C ₄ H ₃ N ₂ O ⁺	112	95	17	11.5	x	x			
+	Creatinine	C00791	C ₄ H ₈ N ₃ O ⁺		114	44	17	10	x	x			
+	<u>Proline</u>	C00148	C ₅ H ₁₀ NO ₂ ⁺	C ₄ H ₈ N ⁺	116	70	11	13.3		x	x		
+	<u>Valine</u>	C00183	C ₅ H ₁₂ NO ₂ ⁺	C ₄ H ₇ ⁺	118	55	11	13.1		x	x		
+	Indole	C00463	C ₈ H ₈ N ⁺	C ₇ H ₇ ⁺	118	91	24	3.2					
+	<u>Betaine</u>	C00719	C ₅ H ₁₁ NO ₂ ⁺	C ₃ H ₈ N ⁺ +?	118.01	58	34	11.7	x	x			
+	Homoserine	C00263	C ₄ H ₁₀ NO ₃ ⁺	C ₂ H ₆ N ⁺	120	44	30	14		x	x		
+	<u>Threonine</u>	C00188	C ₄ H ₁₀ NO ₃ ⁺	C ₃ H ₈ NO ⁺	120	74	11	14.4		x	x		
+	Tris				122	57	19	11.9	x	x	x	x	
+	Cysteine	C00097	C ₃ H ₈ NO ₂ S ⁺	C ₂ H ₃ S ⁺	122.01	59	27	17.5		x	x		
+	<u>Nicotinamide</u>	C00153	C ₆ H ₇ N ₂ O ⁺	C ₅ H ₆ N ⁺	123	80	20	7	x		x	x	
+	Imidazoleacetic acid	C02835	C ₅ H ₇ N ₂ O ₂ ⁺		127	81	13	16			x		
+	<u>Thymine</u>	C00178	C ₅ H ₇ N ₂ O ₂ ⁺	C ₅ H ₄ NO ₂ ⁺	127.01	110	17	7.3	x	x			
+	130-84				130	84	16	13.3		x			
+	DL-Pipecolic acid				131	114	10	16		x			
+	N-acetylputrescine	C02714	C ₆ H ₁₅ N ₂ O ⁺		131.01	114	25	12.6		x	x		
+	<u>(Iso)Leucine</u>	C00407	C ₆ H ₁₄ NO ₂ ⁺	C ₅ H ₁₂ N ⁺	132	86	11	12.4	x	x			
+	creatine	C00300	C ₄ H ₁₀ N ₃ O ₂ ⁺		132.01	90	13	13		x			

Table A2.2 Continued

Polarity	compound	KEGG ID	Parent Formula	Product Formula	Parent Mass	Product Mass	Collision Energy	Expected RT	0-17	17-28	28-35	35-50	Notes
+	4,5-dihydroxy-2,3-pentanedione (DPD+)				133.02	43	15	3.5	x				
+	Ornithine	C00077	$C_5H_{13}N_2O_2^+$	$C_4H_8N^+$	133.01	70	12	15.3		x	x		
+	<u>Asparagine</u>	C00152	$C_4H_9N_2O_3^+$	$C_2H_4NO_2^+$	133.02	74	17	14.7		x	x		
+	<u>Aspartate</u>	C00049	$C_4H_8NO_4^+$	$C_2H_4NO_2^+$	134	74	15	18.2			x	x	
+	Homocysteine	C00155	$C_4H_{10}NO_2S^+$	$C_3H_8NS^+$	136	90	15	12		x			
+	<u>Adenine</u>	C00147	$C_5H_6N_5^+$	$C_5H_3N_4^+$	136.02	119	24	12	x	x			
+	Methylcysteine				136.01	119	10	12.5		x			
+	Histidinol	C00860	$C_6H_{12}N_3O^+$	$C_5H_7N_2^+$	142	95	18	12	x	x			
+	<u>Lysine-b</u>	C00047	$C_6H_{15}N_2O_2^+$	$C_5H_7^+$	147	67	30	15		x	x		
+	Glutamine and Lysine(-a)	C00047 C00064	$C_6H_{15}N_2O_2^+$ $C_5H_{11}N_2O_3^+$		147.01	84	15	15		x	x		Lysine comes after Glutamine
+	<u>Glutamate</u>	C00025	$C_5H_{10}NO_4^+$	$C_4H_6NO_3^+$	148	84	15	18.4			x	x	
+	O-acetyl-L-serine	C00979	$C_5H_{10}NO_4^+$	$C_3H_8NO_3^+$	148.01	106	12	15.7		x	x		
+	<u>Methionine</u>	C00073	$C_5H_{12}NO_2S^+$	$C_5H_9O_2S^+$	150	133	10	13.3		x	x		
+	<u>Guanine</u>	C00242	$C_5H_6N_5O^+$	$C_4H_4N_3O^+$	152	110	18	12.6		x	x		
+	<u>Histidine</u>	C00135	$C_6H_{10}N_3O_2^+$	$C_5H_8N_3^+$	156	110	12	14.8		x	x		
+	Carnitine	C00318	$C_7H_{16}NO_3^+$	$C_4H_7O_3^+$	162	103	18	12.3		x			Lysine degradation, ABC Transporters
+	Phenylalanine	C00079	$C_9H_{12}NO_2^+$	$C_8H_7^+$	166	103	28	11.7	x	x	x		
+	Pyridoxamine	C00534	$C_8H_{13}N_2O_2^+$		169	134	23	13		x			
+	3-methylhistidine	C01152	$C_7H_{12}N_3O_2^+$		170	96	21	15		x	x		
+	Pyridoxine	C00314	$C_8H_{12}NO_3^+$	$C_8H_8NO_3^+$	170.02	134	22	12					Vitamin B6 metabolism
+	1-methylhistidine	C01152	$C_7H_{12}N_3O_2^+$		170.01	124	18	13.7		x			
+	Butyryl-homoserine lactone (C4-HSL)	C11837	$C_8H_{14}NO_3^+$	$C_4H_8NO_2^+$	172	102	10	3.7	x				
+	Arginine	C00062	$C_6H_{15}N_4O_2^+$	$CH_6N_3^+$	175.02	60	14	14.4		x	x		
+	N-acetyl-Ornithine	C00437	$C_7H_{15}N_2O_3^+$	$C_5H_9NO_2^+$	175.01	115	14	13.4		x	x		

Table A2.2 Continued

Polarity	compound	KEGG ID	Parent Formula	Product Formula	Parent Mass	Product Mass	Collision Energy	Expected RT	0-17	17-28	28-35	35-50	Notes
+	Citrulline	C00327	$C_6H_{14}N_3O_3$	$C_6H_{11}N_2O_3^+$	176	159	12	13		x	x		
+	N-Carbamoyl-L-aspartate	C00438	$C_5H_9N_2O_5$	$C_2H_4NO_2^+$	177	74	17	21			x	x	
+	<u>Glucosamine</u>	C00329	$C_6H_{14}NO_5^+$	$C_6H_{12}NO_4^+$	180	162	10	12.9		x	x		
+	<u>Tyrosine</u>	C00082	$C_9H_{12}NO_3$	$C_6H_5^+$	182	77	37	14.2		x	x		
+	Epinephrine	C00788	$C_9H_{14}NO_3^+$		184	166	11	23	x	x	x	x	
+	3-phospho-serine	C01005	$C_3H_9NO_6P$	$C_3H_6NO_2^+$	186	88	10	23.5				x	
+	Tributylamine				186.01	130	18	1					
+	N-acetyl-L-Lysine and Acetyllysine	C02727 C12989	$C_8H_{17}N_2O_3$		189	84	24	6	x	x	x	x	
+	N-acetyl-glutamine		$C_7H_{13}N_2O_4$	$C_5H_8NO_3^+$	189.01	130	15	14.6		x	x	x	
+	N-acetyl-glutamate	C00624	$C_7H_{12}NO_5^+$	$C_4H_6NO^+$	190	84	22	19.6			x	x	
+	Acetylcarnitine	C02571	$C_9H_{19}NO_4^+$		204	85	17	11	x	x			
+	<u>Tryptophan</u>	C00078	$C_{11}H_{13}N_2O_2$	$C_9H_8NO^+$	205	146	16	13.1		x	x		
+	Kynurenine	C00328	$C_{10}H_{13}N_2O_3$		209	146	23	12		x			
+	N-(3-Oxohehexanoyl)homoserine lactone	C11839	$C_{10}H_{16}NO_4$	$C_4H_8NO_2^+$	214	102	13	3.7	x				
+	222-138				222	138	16	12.4	x	x			
+	Flavone	C15608	$C_{15}H_{11}O_2$		223	121	27	5	x				
+	Cystathionine	C02291	$C_7H_{15}N_2O_4S^+$	$C_4H_8NO_2S^+$	223.01	134	11	16.5		x	x		
+	Octanoyl-homoserine lactone		$C_{12}H_{22}NO_3$	$C_4H_8NO_2^+$	228	102	11	3.7	x				
+	Cytidine	C00475	$C_9H_{14}N_3O_5$	$C_4H_6N_3O^+$	244	112	12	12.1	x	x			
+	Biotin (Vitamin H)	C00120	$C_{10}H_{17}N_2O_3S^+$	$C_{10}H_{15}N_2O_2S^+$	245	227	18	15.6		x	x		
+	Deoxyadenosine	C00559	$C_{10}H_{14}N_5O_3$	$C_5H_6N_5^+$	252	136	20	9.7	x	x			
+	Glycerophosphocholine	C00670	$C_8H_{22}NO_6P^+$		258	104	14	12.6		x	x		
+	Acadesine (AICAR without phosphate)		$C_9H_{15}N_4O_5$	$C_4H_4N_3O^+$	259	110	22	6.6	x	x			
+	Glucosamine-6-phosphate	C00352	$C_6H_{15}NO_8P^+$	$C_6H_8NO_2^+$	260	126	15	19.5			x	x	
+	Glucosamine-1P	C06156	$C_6H_{15}NO_8P^+$	$C_6H_{12}NO_4^+$	260.01	162	15	23			x	x	
+	Thiamine (Vitamin B1)	C00378	$C_{12}H_{17}N_4OS^+$	$C_6H_8N_3^+$	265	122	17	3	x	x			
+	Deoxyadenosine	C00559 C05198	$C_{10}H_{14}N_5O_3$		267.1	146	20						

Table A2.2 Continued

Polarity	compound	KEGG ID	Parent Formula	Product Formula	Parent Mass	Product Mass	Collision Energy	Expected RT	0-17	17-28	28-35	35-50	Notes
+	S-Ribosyl-Homocysteine (SRH)	C03539	$C_9H_{18}NO_6S^+$		268	88	29	14	x	x			
+	Adenosine	C00212	$C_{10}H_{14}N_5O_4^+$	$C_5H_6N_5^+$	268.01	136	27	10.5	x	x			
+	Deoxyguanosine	C00330	$C_{10}H_{14}N_5O_4^+$	$C_5H_6N_5O^+$	268.02	152	15	12		x			
+	1-methyladenosine	C02494	$C_{11}H_{16}N_5O_4^+$		282	150	25	11	x	x			
+	Guanosine	C00387	$C_{10}H_{14}N_5O_5^+$	$C_5H_3N_4O^+$	284	135	33	13.1		x			
+	N-(L-Arginino)succinate 5 ⁻	C03406	$C_{10}H_{19}N_4O_6^+$	$C_4H_8N^+$	291	70	35	17.5			x		
+	methylthioadenosine	C00170	$C_{11}H_{16}N_5O_3S^+$	$C_5H_6N_5^+$	298	136	27	8.1	x	x			
+	dCMP	C00239	$C_9H_{15}N_3O_7P^+$	$C_4H_6N_3O^+$	308	112	16	20			x	x	
+	<u>Reduced glutathione</u>	C00051	$C_{10}H_{18}N_3O_6S^+$	$C_5H_8NO_3S^+$	308.01	162	19	20.5			x	x	
+	TMP	C00364	$C_{10}H_{16}N_2O_8P^+$	$C_5H_5O^+$	323	81	17	20.7				x	
+	CMP	C00055	$C_9H_{15}N_3O_8P^+$	$C_4H_6N_3O^+$	324	112	16	22.8			x	x	
+	UMP	C00105	$C_9H_{14}N_2O_9P^+$	$C_5H_5O_2^+$	325	97	12	22			x	x	
+	dAMP	C00360	$C_{10}H_{15}N_5O_6P^+$	$C_5H_6N_5^+$	332	136	21	25	x	x	x		
+	Nicotinamide ribotide	C00455	$C_{11}H_{16}N_2O_8P^+$		335	123	28	17			x		
+	UMP	C00105	$C_9H_{14}N_2O_9P^+$		336	102	25	20.2			x	x	
+	AICAR-MonoPhosphate		$C_9H_{16}N_4O_8P^+$	$C_4H_4N_3O^+$	339	110	30	19.5			x	x	
+	Thiamine-phosphate	C01081	$C_{12}H_{19}N_4O_4PS^+$	$C_6H_8N_3^+$	345	122	13	17			x		
+	dGMP	C00362	$C_{10}H_{15}N_5O_7P^+$	$C_5H_3N_4O^+$	348	135	36	20				x	big peak is from AMP
+	<u>AMP</u>	C00020	$C_{10}H_{15}N_5O_7P^+$	$C_5H_6N_5^+$	348.01	136	21	14.2	x	x	x		
+	IMP	C00130	$C_{10}H_{14}N_4O_8P^+$	$C_5H_5N_4O^+$	349	137	19	25.1			x	x	
+	IS AMP				363.1	146	21						
+	GMP	C00144	$C_{10}H_{15}N_5O_8P^+$	$C_5H_6N_5O^+$	364	152	19	25			x	x	
+	Xanthosine 5'-phosphate	C00655	$C_{10}H_{14}N_4O_9P^+$	$C_5H_5O_2^+$	365	97	11	25				x	
+	<u>Riboflavin</u> (Vitamin B2)	C00255	$C_{17}H_{21}N_4O_6^+$	$C_{12}H_{12}N_4O_2^+$	377	243	24	10.6	x	x			
+	S-adenosyl-L-homoCysteine (SAH)	C00021	$C_{14}H_{21}N_6O_5S^+$	$C_5H_6N_5^+$	385	136	19	14.3	x	x			
+	dCDP	C00705	$C_9H_{16}N_3O_{10}P_2^+$	$C_4H_6N_3O^+$	388	112	25	20			x	x	
+	S-adenosyl-L-methionine (SAM)	C00019	$C_{15}H_{24}N_6O_5S^+$	$C_{10}H_{12}N_5O_3^+$	399	250	13	13.3	x	x			

Table A2.2 Continued

Polarity	compound	KEGG ID	Parent Formula	Product Formula	Parent Mass	Product Mass	Collision Energy	Expected RT	0-17	17-28	28-35	35-50	Notes
+	TDP	C00363	$C_{10}H_{17}N_2O_{11}P_2^+$	$C_5H_5O^+$	403	81	25	21			x	x	The earlier peak at ~19 is not TDP
+	CDP	C00112	$C_9H_{16}N_3O_1P_2^+$	$C_4H_6N_3O^+$	404	112	25	21			x	x	
+	UDP	C00015	$C_9H_{15}N_2O_1P_2^+$	$C_5H_5O_2^+$	405	97	25	21			x	x	The earlier peak at RT19-20 is not UDP
+	ADP	C00008	$C_{10}H_{16}N_5O_{10}P_2^+$	$C_5H_6N_5^+$	428	136	31	21.5			x	x	
+	IDP	C00104	$C_{10}H_{15}N_4O_{11}P_2^+$	$C_5H_5N_4O^+$	429	137	25	23				x	
+	<u>Folate</u>	C00504	$C_{19}H_{20}N_7O_6^+$	$C_{14}H_{11}N_6O_2^+$	442	295	16	23				x	
+	GDP	C00035	$C_{10}H_{16}N_5O_{11}P_2^+$	$C_5H_6N_5O^+$	444	152	30	22.5				x	
+	7,8-dihydrofolate	C00415	$C_{19}H_{22}N_7O_6^+$	$C_7H_8N_5O^+$	444.01	178	30	23				x	
+	5-methyl-tetrahydrofolate	C00440	$C_{20}H_{26}N_7O_6^+$	$C_{15}H_{17}N_6O_2^+$	460	313.1	19	23				x	
+	dCTP	C00458	$C_9H_{17}N_3O_1P_3^+$	$C_4H_6N_3O^+$	468	112	31	24				x	
+	dUTP	C00460	$C_9H_{16}N_2O_1P_3^+$	$C_5H_5O^+$	469	81	18	24				x	
+	TTP	C00459	$C_{10}H_{18}N_2O_{14}P_3^+$	$C_5H_5O^+$	483	81	25	23				x	
+	CTP	C00063	$C_9H_{17}N_3O_1P_3^+$	$C_4H_6N_3O^+$	484	112	21	23				x	
+	UTP	C00075	$C_9H_{16}N_2O_1P_3^+$	$C_5H_5O_2^+$	485	97	30	23				x	
+	dATP	C00131	$C_{10}H_{17}N_5O_{12}P_3^+$	$C_5H_6N_5^+$	492	136	35	24				x	
+	<u>ATP</u>	C00002	$C_{10}H_{17}N_5O_{13}P_3^+$	$C_5H_6N_5^+$	508	136	30	25				x	
+	dGTP	C00286	$C_{10}H_{17}N_5O_{13}P_3^+$	$C_5H_6N_5O^+$	508.01	152	30	25.5				x	dGTP comes after ATP
+	ITP	C00081	$C_{10}H_{16}N_4O_{14}P_3^+$	$C_5H_5N_4O^+$	509	137	25	24				x	
+	ATP-gamma-S	C13742	$C_{10}H_{17}N_5O_{12}P_3S^+$	$C_5H_6N_5^+$	524	136	35	25				x	
+	GTP	C00044	$C_{10}H_{17}N_5O_{14}P_3^+$	$C_5H_6N_5O^+$	524.01	152	37	25.5				x	
+	Diiodothyronine				526	353	29	11	x	x			
+	<u>Oxidized glutathione</u>	C00127	$C_{20}H_{33}N_6O_{12}S_2^+$	$C_8H_{11}N_2O_2S_2^+$	613	231	33	21				x	
+	NAD	C00003	$C_{21}H_{28}N_7O_{14}P_2^+$	$C_{10}H_{16}N_5O_{10}P_2^+$	664	428	30	17			x		
+	NADH	C00004	$C_{21}H_{30}N_7O_{14}P_2^+$	$C_{16}H_{24}N_2O_{13}P_2^+$	666	514	26	19.5		x	x	x	
+	Dephospho-CoA	C00882	$C_{21}H_{36}N_7O_{13}P_2S^+$	$C_{10}H_{15}N_5O_7P^+$	688	348	25	23.5				x	

Table A2.2 Continued

Polarity	compound	KEGG ID	Parent Formula	Product Formula	Parent Mass	Product Mass	Collision Energy	Expected RT	0-17	17-28	28-35	35-50	Notes
+	Cyclic-diGMP	C16463	$C_{20}H_{25}N_{10}O_{14}P_2^+$	$C_5H_6N_5O^+$	691	152	35	23				x	no standard; the last peak among three peaks
+	NADP	C00006	$C_{21}H_{29}N_7O_{17}P_3^+$	$C_5H_6N_5^+$	744	136	48	22				x	
+	NADPH	C00005	$C_{21}H_{31}N_7O_{17}P_3^+$	$C_{21}H_{28}N_6O_{17}P_3^+$	746	729	16	23.5	x			x	
+	CoA	C00010	$C_{21}H_{37}N_7O_{16}P_3S^+$	$C_{11}H_{21}N_2O_3S^+$	768	261	37	24				x	
+	FAD	C00016	$C_{27}H_{34}N_9O_{15}P_2^+$	$C_{10}H_{15}N_5O_7P^+$	786	348	24	25	x	x	x	x	
+	Acetyl-CoA	C00024	$C_{23}H_{39}N_7O_{17}P_3S^+$	$C_{13}H_{23}N_2O_4S^+$	810	303	28	23				x	
+	Propanoyl-CoA	C00100	$C_{24}H_{41}N_7O_{17}P_3S^+$	$C_{14}H_{25}N_2O_4S^+$	824	317	33	25				x	
+	Acetoacetyl-CoA	C00332	$C_{25}H_{41}N_7O_{18}P_3S^+$	$C_{15}H_{15}N_2O_5S^+$	852	345	34	26				x	
+	Malonyl-CoA	C00083	$C_{24}H_{39}N_7O_{19}P_3S^+$	$C_{14}H_{23}N_2O_6S^+$	854	347	34	26				x	

Table A2.3 SRMs with Known Selectivity Issues

Metabolite	SRM	Metabolites with same SRM (Metabolite-RT)	Metabolites the SRM also measures	SRMs that also measure the metabolite
2-oxobutanoate	(-)101-57-11	3-oxobutanoate-11		
3-oxobutanoate	(-)101-57-11	2-oxobutanoate-21.8		
maleate	(-)115-71-11	isoketovalerate-28, fumarate-24.6		
isoketovalerate	(-)115-71-11	maleate-24, fumarate-24.6		
fumarate	(-)115-71-11	maleate-24 , isoketovalerate-28		
methylmalonate	(-)117-73-	Succinate-17.4		
succinate	(-)117-73-	Methylmalonate-24		
oABA	(-)136-92-16	pABA-15		
pABA	(-)136-92-16	oABA-25		
Adipate	(-)145-83-25	2-methylglutarate-28		
2-methylglutarate	(-)145-83-25	Adipate-27.6		
2-oxoglutarate	(-)145-101-11	phenylpropiolate-30.1		adipate, 2-methylglutarate - 1000x
phenylpropiolate	(-)145-101-11	2-oxoglutarate-23.2		adipate, 2-methylglutarate - 1000x
Aminoadipate	(-)160-116-25	Indole-3-carboxylate-32.6		
Indole-3-carboxylate	(-)160-116-25	Aminoadipate-6		
ascorbic acid	(-)175-87-25			2-isopropylmalate
allantoate	(-)175-132-12	carbamoyl aspartate-25		
carbamoyl aspartate	(-)175-132-12	allantoate-9		
myo-inositol	(-)179-161-15		hydroxyphenylpyruvate	hydroxyphenylpyruvate -100x
hydroxyphenylpyruvate	(-)179.01-107-11		myo-inositol	myo-inositol
homocysteic acid	(-)182-80-21			4-pyridoxate -100x
fructose-6-phosphate	(-)259-169		glucose-6-phosphate	hexose-phosphate, glucose-1-phosphate, glucose-6-phosphate, DHAP, glyceraldehyde-3-phosphate

Table A2.3 Continued

Metabolite	SRM	Metabolites with same SRM (Metabolite-RT)	Metabolites the SRM also measures	SRMs that also measure the metabolite
glucose-6-phosphate	(-)259-199-13			hexose-phosphate, glucose-1-phosphate, fructose-6-phosphate
1,3-Glycerate-diphosphate	(-)265-79-35	2,3-glycerate-diphosphate-34		
2,3-Glycerate-diphosphate	(-)265-79-35	1,3-glycerate-diphosphate-31		
Cellobiose	(-)341-179-18	Sucrose-1.7, Trehalose-1.7		
Sucrose	(-)341-179-18	Cellobiose-7, Trehalose-1.7		
Trehalose	(-)341-179-18	Cellobiose-7, Sucrose-1.7		
ADP	(-)426.01-159-25	dGDP-34		
dGDP	(-)426.01-159-25	ADP-31		
ATP	(-)506-159-28	dGTP-37		
dGTP	(-)506-159-28	ATP-35		
UDP-D-galacturonate	(-)579-403-24	UDP-D-glucuronate-34		
UDP-D-glucuronate	(-)579-403-24	UDP-D-galacturonate-34.5		
N-acetylputrescine	(-)131-114-25	N-acetylputrescine-12.6		
DL-Pipecolic acid	(-)131-114-25	DL-Pipecolic acid-16		
Dimethylglycine	(+)104-58-25			
Choline	(+)104.01-60-19			
GABA	(+)104.02-69-20			
Valine	(+)118-55-11			Betaine -100x, Indole -1000x
Betaine	(+)118.01-58-25		Valine-13	
Indole	(+)118.02-91-24		Valine-13	
Imidazoleacetic acid	(+)127-81-13		Thymine-7	
Thymine	(+)127.01-110-17			Imidazoleacetic acid -100x
(Iso)Leucine	(+)132-86-11			Ornithine -100x, DPD -1000x
4,5-dihydroxy-2,3-pentanedione(DPD +)	(+)133-43-25		(Iso)leucine-12.4, Asparagine-14.7	

Table A2.3 Continued

Metabolite	SRM	Metabolites with same SRM (Metabolite-RT)	Metabolites the SRM also measures	SRMs that also measure the metabolite
Ornithine	(+)133.01-70-12		(Iso)leucine-12.4	
Asparagine	(+)133.02-74-17			DPD -10x
Homocysteine	(+)136-90-15		-18.3	
Methylcysteine	(+)136.01-119-10	Adenine-12		
Adenine	(+)136.02-119-24	Methylcysteine		
Lysine(-b)	(+)147-67-30			
Lysine(-a)	(+)147.01-84-15	Glutamine		Glutamate -100x
Glutamine	(+)147.01-84-15	Lysine(-a)-15		
Glutamate	(+)148-84-15		Lysine(-a)-15.0	
3-methylhistidine	(+)170-96-21		1-methylhistidine-13.7	
1-methylhistidine	(+)170.01-124-18			3-methylhistidine -10x
Arginine	(+)175-60-14			N-acetyl-Ornithine -10x
N-acetyl-Ornithine	(+)175.01-115-14		Arginine-14.4	
3-phospho-serine	(+)186-88-10			
Tributylamine	(+)186.01-130-18			
N-acetyl-L-Lysine and Acetyllysine	(+)189-84-24			
N-acetyl-glutamine	(+)189.01-130-15			
Flavone	(+)223-121-27			
Cystathionine	(+)223.01-134-11			
Glucosamine-6-phosphate	(+)260-126-15			
Glucosamine-1P	(+)260.01-162-15			
S-Ribosyl-Homocysteine (SRH)	(+)268-88-29			
Adenosine	(+)268.01-136-27			
Deoxyguanosine	(+)268.02-152-15			
dGMP	(+)348-135-10		AMP-14.2	
AMP	(+)348.01-136-21			dGMP -1000x
GDP	(+)444-152-25			
7,8-dihydrofolate	(+)444.01-178-25			
ATP	(+)508-136-25			
dGTP	(+)508.01-152-25			
ATP-gamma-S	(+)524-136-25			
GTP	(+)524.01-152-25			

Table A7.1 Relevance of Correction Factors Based on Signal: Internal Standard Signal Ratio

Prediction of Error in Measuring (¹³ C)DPD-M1CQ Arising from Naturally Occurring Isotopes of DPD- M1CQ				Prediction of Error in Measuring DPD-M1CQ Arising from ¹² C contaminants in (¹³ C)DPD-M1CQ			
Ratio of Observed SRM 381- 202 Intensity	SRM 382-203 Intensity	Actual SRM 382-203 Intensity	% Over Estimation SRM 382- 203 Intensity	Ratio of Observed SRM 381- 202 Intensity	SRM 382-203 Intensity	Actual SRM 381-202 Intensity	% Over Estimation SRM 381- 202 Intensity
0.9	:	100	99.87	0.9	:	100	800.00
1	:	100	99.86	1	:	100	400.00
2	:	100	99.71	2	:	100	66.67
3	:	100	99.57	3	:	100	36.36
4	:	100	99.42	4	:	100	25.00
5	:	100	99.28	5	:	100	19.05
6	:	100	99.14	6	:	100	15.38
7	:	100	98.99	7	:	100	12.90
8	:	100	98.85	8	:	100	11.11
9	:	100	98.70	9	:	100	9.76
10	:	100	98.56	10	:	100	8.70
20	:	100	97.12	20	:	100	4.17
30	:	100	95.68	30	:	100	2.74
40	:	100	94.24	40	:	100	2.04
50	:	100	92.80	50	:	100	1.63
60	:	100	91.36	60	:	100	1.35
70	:	100	89.92	70	:	100	1.16
80	:	100	88.48	80	:	100	1.01
90	:	100	87.04	90	:	100	0.90
100	:	100	85.60	100	:	100	0.81
110	:	100	84.16	110	:	100	0.73
120	:	100	82.72	120	:	100	0.67
130	:	100	81.28	130	:	100	0.62
140	:	100	79.84	140	:	100	0.57
150	:	100	78.40	150	:	100	0.54
160	:	100	76.96	160	:	100	0.50
170	:	100	75.52	170	:	100	0.47
180	:	100	74.08	180	:	100	0.45
190	:	100	72.64	190	:	100	0.42
200	:	100	71.20	200	:	100	0.40
210	:	100	69.76	210	:	100	0.38
220	:	100	68.32	220	:	100	0.36
230	:	100	66.88	230	:	100	0.35
240	:	100	65.44	240	:	100	0.33
250	:	100	64.00	250	:	100	0.32
260	:	100	62.56	260	:	100	0.31
270	:	100	61.12	270	:	100	0.30
280	:	100	59.68	280	:	100	0.29
290	:	100	58.24	290	:	100	0.28
300	:	100	56.80	300	:	100	0.27

A7.2 Variability in Triplicate Injection

Sample Source: WT <i>V. harveyi</i> grown for 8 h in LM	DPD-M1CQ Intensity, SRM 381-202 (ion counts)	(¹³ C)DPD-M1CQ Intensity, SRM 382-203 (ion counts)	[DPD] (μM)
Replicate 1	1.89 x10 ⁶	1.28 x10 ⁶	18.71
Replicate 2	1.06 x10 ⁶	7.08 x10 ⁵	19.19
Replicate 3	5.31 x10 ⁵	3.65 x10 ⁵	18.41
		Average = 18.77 μM	Range = 0.78 μM

Sample Source: WT <i>V. harveyi</i> grown for 12 h in LM	DPD-M1CQ Intensity, SRM 381-202 (ion counts)	(¹³ C)DPD-M1CQ Intensity, SRM 382-203 (ion counts)	[DPD] (μM)
Replicate 1	5.83 x10 ⁵	8.91 x10 ⁵	7.23
Replicate 2	1.91 x10 ⁵	2.87 x10 ⁵	7.38
Replicate 3	1.67 x10 ⁵	2.59 x10 ⁵	7.12
		Average = 7.24 μM	Range = 0.14 μM

Table A8.1 Detection Parameters for DPD Flux Experiments

Parent Ion <i>m/z</i>	Product Ion <i>m/z</i>	Collision Energy	Heavy Isotopes in Parent Ion	Heavy Isotopes in Fragment Ion
381	202	43	0	0
382	202	43	1	0
382.01	203	43	1	1
383	202	43	2	0
383.01	203	43	2	1
383.02	204	43	2	2
384	202	43	3	0
384.01	203	43	3	1
384.02	204	43	3	2
384.03	205	43	3	3
385	202	43	4	0
385.01	203	43	4	1
385.02	204	43	4	2
385.03	205	43	4	3
385.04	206	43	4	4
386	202	43	5	0
386.01	203	43	5	1
386.02	204	43	5	2
386.03	205	43	5	3
386.04	206	43	5	4
386.05	207	43	5	5
386.06	201	36	5	0
386.07	236	29	5	5
386.08	235	29	5	4
386.09	234	29	5	3
386.1	233	29	5	2
386.11	232	29	5	1
386.12	231	29	5	0
386.13	368	17	5	5
386.14	367	17	5	4
386.15	366	17	5	3
386.16	365	17	5	2
386.17	364	17	5	1
386.18	363	17	5	0

Table A9.1 Changes in Gene Expression DPD : Cyclohexanone

Gene	Gene Name	Fold Change	p-value
4930570C		2.46	0.01153
9330155C		1.45	0.04223
9630027A		1.45	0.04924
ABCB6	ATP-binding cassette, sub-family B (MDR/TAP), member 6	1.79	0.02562
ABHD4	abhydrolase domain containing 4	1.65	0.00778
ACSS2	acyl-CoA synthetase short-chain family member 2	3.81	0.00220
AFG3L2	similar to AFG3(ATPase family gene 3)-like 2 (yeast); AFG3(ATPase family gene 3)-like 2 (yeast)	1.37	0.03672
AKR1A4	aldo-keto reductase family 1, member A4 (aldehyde reductase)	1.55	0.01153
AKR1B8	aldo-keto reductase family 1, member B8	3.21	0.00136
ALAS1	aminolevulinic acid synthase 1	2.07	0.01240
ALDH2	aldehyde dehydrogenase 2, mitochondrial	1.46	0.04883
ALOX5AP	arachidonate 5-lipoxygenase activating protein	1.72	0.01240
		1.84	0.04151
AMPD3	adenosine monophosphate deaminase 3	2.64	0.01649
BC028528	cDNA sequence BC028528	1.76	0.02006
BLVRB	biliverdin reductase B (flavin reductase (NADPH))	4.37	0.00001
		5.25	0.00081
CALR3	calreticulin 3	2.52	0.00111
		3.59	0.00076
CASP1	caspase 1; hypothetical protein LOC100044207	1.76	0.00658
CAT	catalase	3.99	0.00111
CBR3	carbonyl reductase 3	3.66	0.00136
		3.87	0.00479
CCL4	chemokine (C-C motif) ligand 4	-1.88	0.01153
		-1.83	0.03629
CLEC4D	C-type lectin domain family 4, member d	1.64	0.01143
CREG1		3.48	0.00112
CSK	c-src tyrosine kinase	-1.34	0.04011
CXCR4	chemokine (C-X-C motif) receptor 4	1.99	0.00563
CYB5	cytochrome b-5	2.63	0.00171
		3.57	0.00847
DAPK1	death associated protein kinase 1	1.42	0.04374
DHX58	DEXH (Asp-Glu-X-His) box polypeptide 58	-1.76	0.00602
DNMT3L	similar to DNA cytosine-5 methyltransferase 3-like protein; DNA (cytosine-5-)-methyltransferase 3-like	2.59	0.02537
DNMT3L		2.92	0.01143
EIF2AK2	eukaryotic translation initiation factor 2-alpha kinase 2	-1.63	0.04883
EMR1	EGF-like module containing, mucin-like, hormone receptor-like sequence 1	1.73	0.03579

Table A9.1 Continued

Gene	Gene Name	Fold Change	p-value
ESD	similar to esterase D/formylglutathione hydrolase; esterase D/formylglutathione hydrolase; predicted gene 5456; predicted gene 2904	1.39	0.02876
		3.14	0.00111
FAM129A	family with sequence similarity 129, member A	1.53	0.04905
G6PDX	glucose-6-phosphate dehydrogenase X-linked	2.21	0.00778
GCLM	glutamate-cysteine ligase, modifier subunit	7.57	0.00001
GSS	glutathione synthetase	1.53	0.00854
		1.56	0.01025
HMOX1	heme oxygenase (decycling) 1	2.28	0.04934
IFITM1	interferon induced transmembrane protein 1	-1.57	0.04883
IGF1	insulin-like growth factor 1	2.27	0.00111
		2.42	0.00112
IRF9	interferon regulatory factor 9	-1.62	0.03769
IRGM1	immunity-related GTPase family M member 1	-1.78	0.01143
ITPR2	inositol 1,4,5-triphosphate receptor 2	1.99	0.01143
LAYN	layilin	2.34	0.00180
LIP1	lysosomal acid lipase A	2.22	0.01469
		2.44	0.00433
LOC10004		-2.85	0.02935
		1.93	0.01601
LOC22416		2.25	0.00112
LOC22448		1.59	0.04374
LOC23488		3.19	0.00111
LOC38070		-1.97	0.00526
LOC38606		2.62	0.00112
LOC63072		2.85	0.02128
LOC67731		1.76	0.00854
LYZL4	lysozyme-like 4	1.57	0.04151
MGST1	microsomal glutathione S-transferase 1	2.13	0.01100
MGST2	microsomal glutathione S-transferase 2	2.63	0.00112
MITD1	microtubule interacting and transport, domain containing 1	-1.47	0.02935
NCAPH2	non-SMC condensin II complex, subunit H2	-1.50	0.01153
NRP1	neuropilin 1	1.93	0.04164
OAS2	2'-5' oligoadenylate synthetase 2	-2.04	0.00602
OSGIN1	oxidative stress induced growth inhibitor 1	1.72	0.00973
PANX1	pannexin 1	1.98	0.00517
		2.10	0.00944
PARP14	poly (ADP-ribose) polymerase family, member 14	-1.92	0.02528
PDLIM4	PDZ and LIM domain 4	2.39	0.00060

Table A9.1 Continued

Gene	Gene Name	Fold Change	p-value
PRDX1	peroxiredoxin 1; predicted gene 7204	2.14	0.01621
PRKAR2B	protein kinase, cAMP dependent regulatory, type II beta	3.72	0.03284
RIOK3	RIO kinase 3 (yeast)	1.62	0.02899
SAMD9L	sterile alpha motif domain containing 9-like	-1.81	0.01143
SERPINB6	serine (or cysteine) peptidase inhibitor, clade B, member 6	1.61	0.03571
SLC11A1	solute carrier family 11 (proton-coupled divalent metal ion transporters), member 1	3.28	0.00076
		3.92	0.00115
		4.12	0.00221
		4.25	0.00180
SLC40A1	solute carrier family 40 (iron-regulated transporter), member 1	5.86	0.00280
SLC7A11	solute carrier family 7 (cationic amino acid transporter, y+ system), member 11	2.24	0.00256
SQRDL	sulfide quinone reductase-like (yeast)	2.35	0.01143
SRXN1	sulfiredoxin 1 homolog (S. cerevisiae)	3.41	0.00017
		4.31	0.00407
		4.70	0.00346
TALDO1	transaldolase 1	2.23	0.01153
TBC1D23	TBC1 domain family, member 23	1.57	0.02617
TMC6	transmembrane channel-like gene family 6	1.78	0.04687
TMEM43	transmembrane protein 43	1.63	0.04883
		1.65	0.04883
TNFAIP2	tumor necrosis factor, alpha-induced protein 2	1.71	0.01143
		1.89	0.03070
TREML4	triggering receptor expressed on myeloid cells-like 4	1.70	0.01100
		1.91	0.01358
TUBB2B	tubulin, beta 2a, pseudogene 2; tubulin, beta 2B	2.50	0.01008
UBAP1	ubiquitin-associated protein 1	1.50	0.01143
UBE2E2	ubiquitin-conjugating enzyme E2E 2 (UBC4/5 homolog, yeast)	1.47	0.04191
UGT1A10	similar to UDP glycosyltransferase 1 family polypeptide A13;	1.57	0.01849
UGT1A6B	similar to UDP glycosyltransferase 1 family polypeptide A13;	1.55	0.01143
USE1	unconventional SNARE in the ER 1 homolog (S. cerevisiae)	-1.40	0.03769
USP18	ubiquitin specific peptidase 18; similar to ubiquitin specific protease UBP43	-3.36	0.01153

Table A9.2 Changes in Gene Expression DPD + LPS : Cyclohexanone + LPS

Gene	Gene Name	Fold Change	p-value
4930570C		2.68	0.02292
ACSS2	acyl-CoA synthetase short-chain family member 2	2.51	0.03514
AKR1A4	aldo-keto reductase family 1, member A4 (aldehyde reductase) (EC:1.1.1.2)	1.57	0.02544
AKR1B8	aldo-keto reductase family 1, member B8	2.51	0.01501
BLVRB	biliverdin reductase B (flavin reductase (NADPH))	2.63	0.00047
BLVRB		3.10	0.01220
CALR3	calreticulin 3	2.32	0.00480
CALR3		3.09	0.00282
CAT	catalase	2.36	0.02575
CBR3	carbonyl reductase 3	2.38	0.02589
CBR3		3.19	0.02544
CCL7	chemokine (C-C motif) ligand 7	-5.53	0.04789
CREG1	cellular repressor of E1A-stimulated genes 1	2.51	0.01501
CYB5	cytochrome b-5	2.30	0.01220
ENPP2	ectonucleotide pyrophosphatase/phosphodiesterase 2	1.44	0.04478
ENSMUSG0		2.87	0.00480
GCLM	glutamate-cysteine ligase, modifier subunit	4.22	0.00045
GSS	glutathione synthetase	1.42	0.04789
HMOX1	heme oxygenase (decycling) 1	2.72	0.04160
ITPR2	inositol 1,4,5-triphosphate receptor 2	1.98	0.02667
LAYN	layilin	2.11	0.01220
LIF	leukemia inhibitory factor	-1.46	0.04160
LOC23488		2.11	0.02486
LOC38606		1.90	0.02486
MGST2	microsomal glutathione S-transferase 2	2.45	0.00480
NRP1	neuropilin 1	2.33	0.02575
PANX1	pannexin 1	1.89	0.02009
PRDX1	peroxiredoxin 1; predicted gene 7204	2.41	0.02340
SLC40A1	solute carrier family 40 (iron-regulated transporter), member 1	3.64	0.03367
SRXN1	sulfiredoxin 1 homolog (S. cerevisiae)	2.38	0.00282
SRXN1		3.06	0.03484
SRXN1		3.07	0.04096
TALDO1	transaldolase 1	2.31	0.02544
UGT1A10	similar to UDP glycosyltransferase 1 family polypeptide A13	1.79	0.01501

VITA

Jessica Gooding began her undergraduate studies at The College of William and Mary in Williamsburg, VA and then moved in 2005 to North Carolina State University where she received her BS in 2007. She worked 2 years in a molecular pharmacology lab at Inspire pharmaceuticals during her junior and senior years in Raleigh. She is currently a fifth year student in Dr. Shawn Campagna's lab where she develops and applies bioanalytical techniques to study a number of biological phenomena ranging from carbon cycling in the ocean, to bacterial communication, and obesity.