Glyphosate as a Source of Phosphorus for Bacteria

Selection of an Enrichment Culture for Glyphosate Degradation, and Analysis of the C-P Lyase Pathway

by

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A thesis

presented to the University of Waterloo

in fulfillment of the

thesis requirement for the degree of

Doctor of Philosophy

in

Biology

Waterloo, Ontario, Canada, 2024

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Author's Declaration

This thesis consists of material all of which I authored or co-authored: see Statement of Contributions included in the thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

I understand that my thesis may be made electronically available to the public.

Statement of Contributions

Chapters 2, 3, along with portions of the abstract, and portions of the general introduction, are adapted and expanded from a draft paper. The draft was written by me and edits were suggested by Geoff Horsman, Trevor Charles, Mighui Ma, and Afshan Ardalan, as they are collaborators on the full paper. Portions entirely written by someone other than me (such as the procedure and results for the contaminating phosphorus tests, written by Afshan Ardalan) have been removed from this work. The edited draft paper served as the basis for Chapters 2 and 3 of this thesis, and both chapters have since been expanded by me. The draft also served as a starting point for the abstract and general introduction. I greatly appreciate their editing and intellectual contributions to these chapters, and look forward to further collaboration on the path to publishing the work in those chapters.

Section 3.2.3, DNA isolation, sequencing, and species identification was adapted by me from a report produced by Metagenom Bio Life Science Inc (Trevor Charles, Michael Lynch, and Jiujun Cheng). Sequencing and species identification in Chapter 3 was performed by Metagenom Bio Life Science Inc. Code used to analyse species abundance, alpha diversity, and beta diversity was originally written by Michael Lynch and Zjardyn Liera-Hood, and further adapted and expanded by me.

Chapter 4, short read Illumina sequencing was performed by the Wastewater team of the Charles lab; Alyssa Overton, Shiv Naiik, Samran Prasla, Jennifer Knapp, and Yemu Hungwe. Long read sequencing of *A. pusense* RIM-Id RfA was performed by the McGill

University and Genome Quebec Innovation Centre, remaining long read sequencing was performed by myself and Aranksha Thakor.

The remainder of this thesis was written entirely by me, with editing suggestions by Trevor Charles.

Abstract

Glyphosate is one of the world's most widely used herbicides, the active ingredient in Roundup as well as many generic herbicide brands. Though it is one of the safer herbicides, its ubiquity necessitates further study of the pathways that bacteria can use to degrade it. This study investigates the ability of bacteria to break the strong carbon-phosphorus bond of glyphosate. The carbon-phosphorus bond has the highest activation energy of the bonds within glyphosate, and breaking this bond is therefore a rate-limiting step of complete glyphosate mineralization. By optimizing a defined media and supplementing with glyphosate as the only source of phosphorus, the ability of bacterial species to grow on glyphosate as the sole source of phosphorus can be tested and compared to their growth when easily accessible ortho-phosphate is provided. This defined media was used to create an enrichment culture to study which species are selected for by glyphosate as the sole source of phosphorus, and compare them to cultures selected on easily accessible ortho-phosphate. Glyphosate as sole source of phosphorus selected for a different suite of species, especially those in the Rhizobiaceae family, than ortho-phosphate selected for. The Rhizobiaceae genus *Agrobacterium* was found to contain particularly good degraders of glyphosate, and a deletion and replacement experiment revealed that the C-P lyase pathway is required for glyphosate degradation in an *Agrobacterium pusense* isolate. Genome and growth comparisons between *A. pusense*, *A. tumefaciens*, and *A. fabrum* isolates revealed them all to be able to degrade glyphosate, and that they share very similar C-P lyase operons, despite having significant heterogeneity in their genomes. Additionally, a commercial enzyme linked

immunosorbent assay was used to quantify glyphosate degradation. This work adds support to the theory that C-P lyase is required for glyphosate degradation in *Agrobacterium*, and sets the stage for additional research into glyphosate degradation pathways by bacteria.

Acknowledgements

Thank you to my supervisor, Trevor Charles, for giving the opportunity to work in his lab. I am grateful to have had the freedom to pursue this research without having to worry about financial constraints. I hope I have grown as a scientist and a person over the course of this PhD. In addition, thank you to NSERC for funding this research.

Thank you to my committee, Geoff Horsman, Andrew Doxey, and Laura Hug. Your advice throughout this project was critical to its development. I greatly appreciate the time you took to help me.

Thank you to collaborating labs, in particular the Horsman lab of Wilfrid Laurier University, including Minghui Ma and Afshan Ardalan. I hope this work helps to get the papers we have been collaborating on published and out into the world.

Thank you to my labmates, both those working at the university and working at Parkside. Thank you to Michael Lynch and Zjardyn Liera-Hood for their help with bioinformatics, your collaboration was critical to the analysis presented in Chapter 3. Thank you also to Jiujun Cheng, Aranksha Thakor and Shirley Wong for help with the genomic library presented in Chapter 4. Thanks also goes to Jiujun Cheng, the Metagenom team, Alyssa Overton, and the wastewater team for the Illumina sequencing presented here. Additional thanks goes to Alyssa for keeping the lab running smoothly in addition to all of her research responsibilities. Thank you to all of my other labmates, for providing an enjoyable working environment. Thank you to the work-study students who allowed me to focus on the research and not the dishes.

Speaking of Alyssa, thank you to my friends for keeping me sane through this experience. In particular, thank you to Alyssa, Alex, Delaney, and Max, see you at Grad House when this is over.

Thank you to all of my teachers and professors. In particular, thank you to Mrs. Henderson, Mr. Reid, Ms. Milne, Mr. Storm, Dr. Moffatt, and Dr. Chuong. All of you played an important roll in getting me to this point.

Thank you to my family, especially my parents, my sister Madison, Oma, Opa, Grandpa, and Grandma. Thank you for raising me to be the person I am today and for supporting me through this very long PhD process. Thank you Oma for the weekly calls.

2Timothy 4:7

Dedication

I dedicate this thesis to my parents, John and Wilma. Thank you for raising me to value education, for putting me in Scouts, taking me camping, and giving me free reign on the property. All of which instilled in me a Naturalist's love of science and the outdoors.

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List of Abbreviations

AMPA	Aminomethylphosphonic acid
ANI	Average nucleotide identity
ASV	Actual sequence variant
ATP	Adenosine triphosphate
BLAST	Basic Local Alignment Search Tool
BV-BRC	Bacterial and Viral Bioinformatics Resource Center
Cm	Chloramphenicol
EC	Enzyme Commission (number)
ELISA	Enzyme linked immunosorbent assay
EPSPS	5-enolpyruvylshikimate-3-phosphate synthase
GTDB	Genome Taxonomy Database
Km	Kanamycin
LB	Lysogeny broth
Nal	Nalidixic acid
Nm	Neomycin
Pi	Inorganic phosphate
RAST	Rapid Annotation using Subsystem Technology
Rif	Rifampicin
Sm	Streptomycin
Spec/Sp	Spectinomycin
Tet	Tetracycline
TSA/TSB	Tryptic soy agar/broth
US EPA	United States Environmental Protection Agency

Chapter 1
General Introduction

1.1 Glyphosate in agriculture

Glyphosate is an effective and widely used broad spectrum herbicide. Since its discovery in 1974 it has been used worldwide and is one of the least toxic pesticides to birds and mammals, being less toxic even than sodium chloride (Gill et al., 2017). It is also considered non-carcinogenic by virtually all regulatory bodies, including the US EPA and Health Canada (Gill et al., 2017; Health Canada Pest Management Regulatory Agency, 2017). With the advent of crops genetically modified for glyphosate resistance, farmers now effectively kill a wide range of weeds with a single herbicide that does not harm the crop plants. In an effort to reduce soil loss, it has been used on farms both small and large as a means to control weeds that does not require soil destroying tillage (Locke et al., 2015; Thierfelder et al., 2013). However, under some conditions, glyphosate may not degrade sufficiently and this may cause build up in soils or leach into local watercourses (Soracco et al., 2018; Van Stempvoort et al., 2016). Low soil pH and low phosphorus can lead to glyphosate adsorbing to soil particles and becoming recalcitrant to degradation (Mamy et al., 2005). Glyphosate leaching into lower soil layers can also slow its degradation (Veiga et al., 2001). Understanding, discovering, and engineering degradation pathways and glyphosate degrading organisms may offer a solution to degrade glyphosate before it leaches into the wider environment.

1.2 Glyphosate mode of action

Glyphosate interacts with bacteria via two main mechanisms; it can inhibit bacterial growth, or it can be used as a nutrient source. When glyphosate acts as a growth inhibitor, bacterial survival requires either glyphosate detoxification or resistance of the target enzyme in the aromatic amino acid synthesis pathway (Tian et al., 2010). As a nutrient source, glyphosate can be a source of hydrocarbons, nitrogen, and phosphorus. This study focuses on glyphosate as a source of phosphorus as a necessary step to complete mineralization.

In both plants and bacteria, glyphosate inhibits the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS; EC 2.5.1.19) of the shikimic acid pathway (Figure 1.1) (Pollegioni et al., 2011). EPSPS is inhibited when glyphosate binds to the same site as the intended substrate, phosphoenolpyruvate (Pollegioni et al., 2011). The shikimic acid pathway furnishes aromatic amino acids and consequently most aromatic compounds in the majority of plants, bacteria, fungi, and archaea (Tian et al., 2010). Inhibition of EPSPS by glyphosate leads to death of plants and bacteria unless resistance mechanisms are deployed.

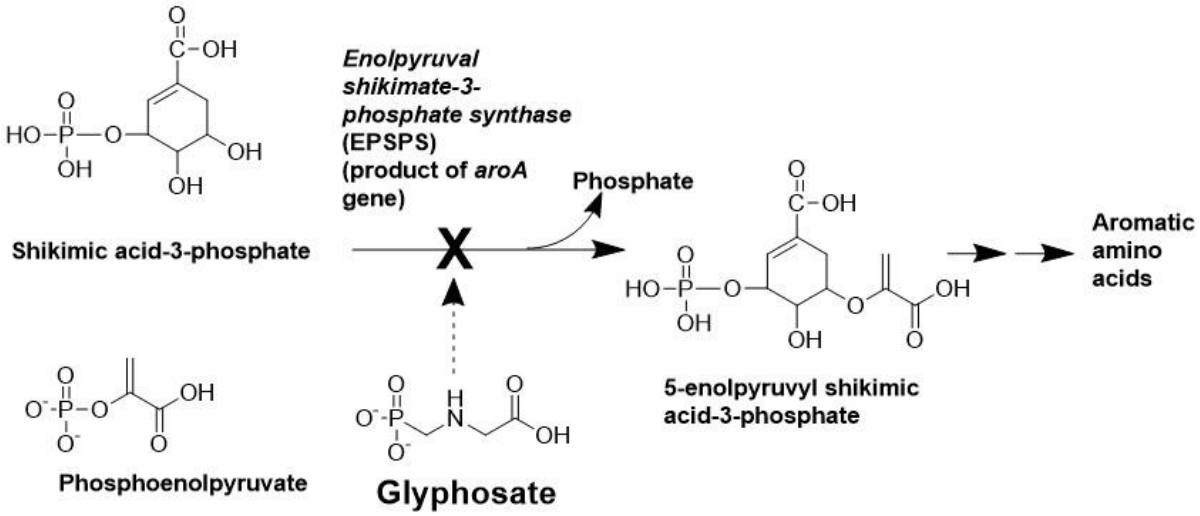


Figure 1.1 Glyphosate Mode of Action: Glyphosate shuts down the aromatic amino acid pathway by inhibiting the enolpyruval shikimate-3-phosphate synthase enzyme (EPSPS), which is coded for by the *aroA* gene. This prevents the production of 5-enolpyruval shikimic acid-3-phosphate, which prevents the aromatic amino acid pathway from proceeding. Figure based on **Pollegioni et al., 2011**.

1.3 Resistance mechanisms

Among glyphosate resistance mechanisms, target modification (i.e. resistant EPSPS) is common in bacteria. EPSPS can be categorized based on sequence as class I, class II, class III, or class IV (Leino et al., 2021). Classes I and II are common with the former generally susceptible and the latter resistant to glyphosate; variably resistant classes III and IV are very rare and significantly different from a sequence perspective (Leino et al., 2021). Classes III and IV are found in less than 1% of eukaryotic species and less than 4% of bacterial and archaeal species (Leino et al., 2021; Pollegioni et al., 2011). While class II EPSPS is always resistant to glyphosate, resistance can be conferred upon normally susceptible class I EPSPS by a single amino acid change. For example, a G96A substitution in the class I EPSPS of *Escherichia coli* causing a methyl group to protrude into the phosphoenolpyruvate binding site and disclude glyphosate while still permitting phosphoenolpyruvate to bind with lower affinity (Pollegioni et al., 2011). This resistance however comes at a cost of decreased

enzyme efficiency (Pollegioni et al., 2011). Class II EPSPS do not suffer decreased enzyme efficiency as a consequence of their glyphosate resistance (Pollegioni et al., 2011)

The canonical class I EPSPS is found in *E. coli*, and also tends to be found within other Enterobacteriaceae (including *Klebsiella,* and *Enterobacter)* (Rainio et al., 2021). The canonical class II EPSPS is from *Agrobacterium* sp. CP4, and this was one of the sequences used to engineer glyphosate resistant crop plants (Massot et al., 2021; Pollegioni et al., 2011). Despite the canonical class II EPSPS coming from *Agrobacterium*, a member of the Rhizobiaceae family, recent studies have concluded that Rhizobiaceae contains both members with class I and members with class II EPSPS (Massot et al., 2021). However, the class I EPSPS within the studied Rhizobiaceae were from isolates resistant to glyphosate and were found to have only 40% amino acids matching *E. coli* class I EPSPS (Massot et al., 2021). Class II EPSPS can also be found within the *Pseudomonas, Acinetobacter,* and *Xanthomonas* genera, which are more closely related to the class I harbouring Enterobacteriaceae than to the Rhizobiaceae that contains class II harbouring isolates (Massot et al., 2021; Rainio et al., 2021). Clearly, EPSPS classes do not fall neatly along phylogenetic lines.

Alternative mechanisms for bacterial glyphosate resistance are shown in Figure 1.2 and involve glyphosate degradation or modification. Two mechanisms are known: (i) acetylation by glyphosate acetyltransferase, or (ii) oxidation by glycine oxidase or glyphosate oxidoreductase (Ma et al., 2024; Pollegioni et al., 2011). Both oxidation mechanisms produce aminomethylphosphonic acid (AMPA) (Pollegioni et al., 2011). Alternatively, the carbon phosphorus bond of glyphosate may be broken by the C–P lyase system, however this is a slow and energy intensive process and is unlikely to act as an effective resistance route (Kamat et al., 2020). Unlike many other phosphonates that do not block metabolic pathways, glyphosate degradation requires bacteria to first protect their aromatic amino acid pathway before glyphosate can be used as a source of phosphorus. The detoxification of glyphosate can be a preliminary step or can be completely separate from its degradation.

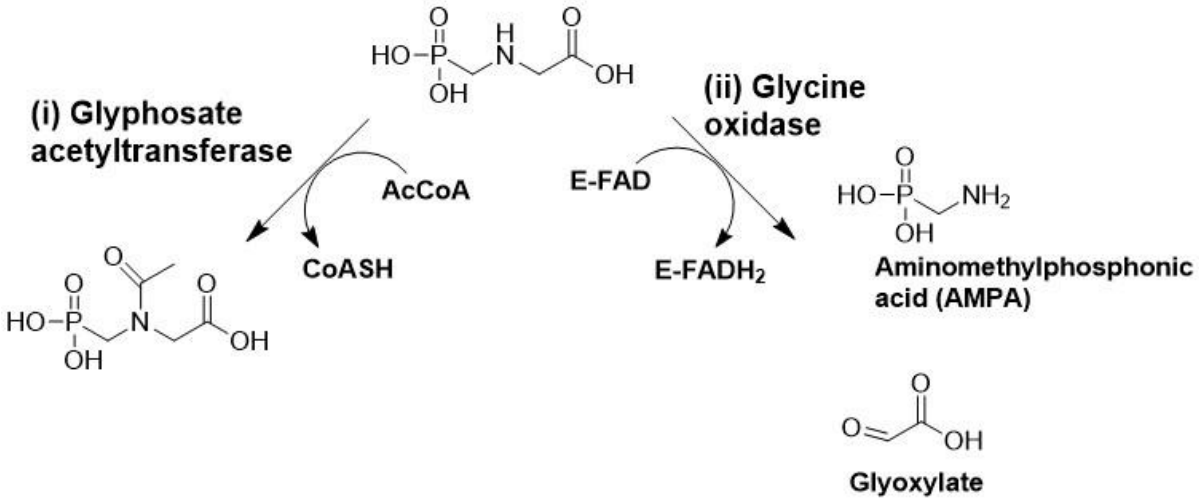


Figure 1.2 Glyphosate Resistance Mechanisms: Glyphosate can be modified so that it no longer inhibits its target enzyme. This is generally performed by acetylation (i) or oxidation (ii). For the oxidative pathway, glycine oxidase is shown which uses FAD as the electron acceptor, a glyphosate oxidase pathway also exists which uses O_2 directly. Figure based on **Pollegioni et al., 2011**.

1.4 Glyphosate structure and bacterial C-P bond breakage

Glyphosate is a phosphonate compound, meaning that it contains a strong carbon-phosphorus bond. This contrasts with the easily broken C-O-P bond structure present in molecules such as adenosine monophosphate. The phosphonate bond of glyphosate requires much higher activation energy to break than carbon-carbon bonds, and therefore acts as the limiting step in full mineralization of glyphosate (Stosiek et al., 2020). Additionally, partial mineralization of glyphosate can lead to aminomethylphosphonic acid (AMPA) production which still contains the difficult to break carbon-phosphorus bond (Stosiek et al., 2020). To avoid this, glyphosate can be selected for use by bacteria as the sole source of phosphorus which required the bacteria to break the carbon-phosphorus bond in order to liberate phosphorus.

The carbon phosphorus bond can be cleaved either before, or after conversion to AMPA (Stosiek et al., 2020).

Three generalized mechanisms are known to break carbon-phosphorus bonds: hydrolytic cleavage, oxidative cleavage, and radical (C-P lyase) cleavage. Of these only radical cleavage is presently known to degrade the carbon-phosphorus bond of glyphosate (Kamat et al., 2020; Stosiek et al., 2020). Radical cleavage uses the energy-intensive C-P lyase system, which accepts a broader range of substrates than either the oxidative or hydrolytic cleavage mechanisms (Kamat et al., 2020). The C-P lyase pathway is encoded by the *phn* operon, canonically consisting of *phnCDEFGHIJKLMNOP* in *E. coli*, though variations in copy number and structure exist in other bacterial species (Hove-Jensen et al., 2014; Kamat et al., 2020). PhnCDE forms an ATP binding cassette type transporter complex that imports phosphonates, which are recognized by the PhnD component (Kamat et al., 2020). PhnGHIJKLM forms a membrane associated multi-enzyme complex (the C-P lyase complex) which performs the actual bond breaking pathway (Figure 1.3) (Stosiek et al., 2020). When cleaving glyphosate, cleavage is generally understood to occur through direct cleavage of glyphosate or through first pre-processing glyphosate to AMPA (Stosiek et al., 2020). The PhnI(GHL) portion of the complex substitutes the adenine moiety of ATP with glyphosate/AMPA by its phosphonyl group. PhnM catalyzes the release of PPi from the ATP-glyphosate molecule. The radical SAM enzyme, PhnJ, then cleaves the C–P bond of glyphosate/AMPA by forming a cyclic ribosyl phosphate (Kamat et al., 2020). The cyclic phosphate is hydrolyzed by PhnP to a-D-ribosyl-1,5-diphosphate, which is phosphorylated by

PhnN to create 5-phospho-α-D-ribosyl-1-pyrophosphate (a second phosphate group is added to the phosphate at the carbon 1 position) (Kamat et al., 2020). This regenerates 5-phospho-α-D-ribosyl-1-pyrophosphate that can be returned to the cycle to assist in breaking another C-P bond. PhnO uses acetyl-CoA to *N*-acetylate aminoalkylphosphonates such as the glyphosate degradation product AMPA prior to their entry into the C-P lyase system, likely to neutralize the positive charge of the amino group.

Though this is the canonical C-P lyase system, variations exist (Hove-Jensen et al., 2014). Of particular relevance to this study, members of the *Agrobacterium* genus generally do not have *phnP*. In its place, *Agrobacterium* have *duf1045* which may substitute for PhnP though its function is unknown (Hove-Jensen et al., 2014; Masotti et al., 2021). The gene *duf1045*, as well as a lack of *phnP*, is also found in other Rhizobiaceae members, including *Ensifer meliloti* and *Ochrobactrum anthropi* (Hove-Jensen et al., 2014). Additionally, Rhizobiaceae tend to have a second copy of *phnE*. Their transporter genes are arranged as *phnCDE$_1$E$_2$* rather than as *phnCDE* in *E. coli* (Hove-Jensen et al., 2014).

Glyphosate specifically can take one of two routes through the C-P lyase, direct degradation by C-P lyase, or pre-processing to AMPA by glyphosate oxidoreductase followed by C-P lyase (Stosiek et al., 2020). Direct glyphosate degradation by C-P lyase cleaves directly at the carbon-phosphorus bond, yielding Pi and sarcosine (Stosiek et al., 2020). When using the indirect route glyphosate is first cleaved at the carbon nitrogen bond at the opposite side of the nitrogen from phosphonate, which yields AMPA and glyoxylate (Pollegioni et al., 2011; Stosiek et al., 2020). The AMPA can then be degraded through the

C-P lyase pathway. Additionally, cleaving glyphosate into AMPA prevents it from inhibiting the aromatic amino acid pathway, rendering it non-toxic to otherwise sensitive bacterial strains (Pollegioni et al., 2011). It may be that some strains are cleaving glyphosate to AMPA not as a pre-processing step for C-P lyase, but instead as a detoxification process necessary for them to live long enough to take advantage of the phosphorus provided by glyphosate.

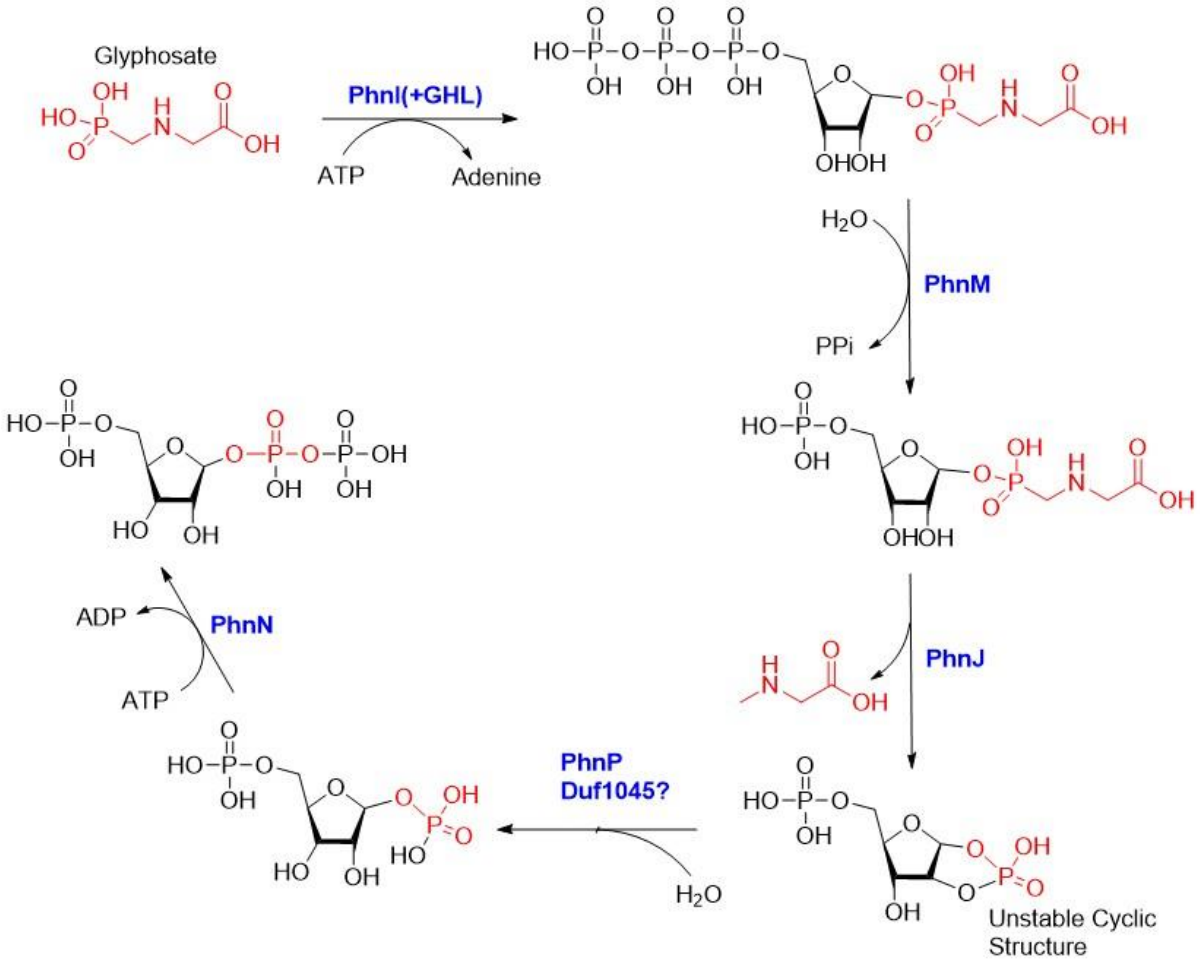


Figure 1.3 C-P Lyase Pathway of *Agrobacterium*: After import (likely by $PhnCDE_1E_2$), glyphosate can be broken down using the carbon-phosphorus lyase system. Shown here is the direct pathway, however glyphosate may first be cleaved into aminomethylphosphonic acid before being further split by this pathway. It is important to note that PhnP is used to split the unstable cyclic structure created by PhnJ, however PhnP is missing in *Agrobacterium* species. *Duf1045*, a common gene within the Rhizobiaceae C-P lyase operon, may substitute for this function. Figure based on **(Horsman & Zechel, 2017; Stosiek et al., 2020)**

1.5 C-P lyase operon structure

The first C-P lyase was discovered in *E. coli*, and is canonical coded for by the

phnCDEFGHIJKLMNOP operon (Stosiek et al., 2020). Expression is induced under

phosphorus starvation conditions by the two component regulatory system, PhoR/PhoB (Stosiek et al., 2020). In *E. coli*, functions are generally grouped, with *phnCDE* encoding the phosphonate transport system, *phnF* encoding a repressor, *phnGHIJKLM* encoding the C-P lyase complex that performs bond activation and cleavage (with PhnJ performing the actual C-P bond cleavage step), and *phnNOP* performing accessory but essential functions within the C-P lyase pathway (Stosiek et al., 2020). In other species, different arrangements and different copy numbers predominate, though functions are generally still grouped (Hove-Jensen et al., 2014). For example, *Agrobacterium radiobacter* and *A. tumefaciens* have the arrangement *phnFGHIJKLO- phnCDE$_1$E$_2$ -duf1045-phnMN* (Hove-Jensen et al., 2014; Masotti et al., 2021)**.**

1.6 Aims

This study aims to examine glyphosate degradation by bacteria. Specifically, bacteria is assessed for its ability to break the strong carbon-phosphorus bond of glyphosate in order to liberate usable Pi. The best way to ensure that the carbon-phosphorus bond is broken is to select for bacteria capable of using glyphosate as sole source of phosphorus.

In order to definitively demonstrate that bacteria are using glyphosate as sole source of phosphorus requires a media that provides all of the conditions necessary for bacterial growth and is sufficiently free of contaminating phosphorus to preclude growth if Pi is not liberated from glyphosate. Chapter 2 of this work aims to create and optimize the media

along with a set of handling procedures and controls that are necessary to assess whether an isolate uses glyphosate as a sole source of phosphorus.

Because glyphosate is a herbicide commonly used in agriculture, it has the potential to act as a source of phosphorus for bacterial communities exposed to it. While studies have been performed to assess the effects of glyphosate on soil communities in the field, Chapter 3 aims to examine the effects of glyphosate as a source of phosphorus in lab grown enrichment cultures and to examine the difference between communities selected for growth on either glyphosate or on an easily accessible phosphorus salt (NaH_2PO_4). Comparisons are achieved through the creation of enrichment cultures using the aforementioned media and through identifying the species within those cultures through sequencing of the 16S rRNA gene.

To go beyond community analysis, Chapter 4 aims to examine specific glyphosate degrading bacteria and the mechanisms used by those bacteria for glyphosate degradation. To that end, *Agrobacterium* isolates are assessed for glyphosate degradation, sequenced, and their genomes assembled and compared. Particular focus is paid to the C-P lyase operon. To elucidate the necessity of the C-P lyase operon, a gene deletion and replacement experiment is performed using *Agrobacterium pusense* RIM-Id RfA. The wild type, knockout, and complemented *A. pusense* RIM-Id RfA are assessed for growth on glyphosate. Glyphosate degradation is also measured directly using a quantitative enzyme linked immunosorbent assay.

Chapter 2

Improved Minimal Media with a Defined Phosphorus Source Suitable for Query and Selection of Glyphosate Degradation

2.1 Introduction

2.1.1 Selecting for effective glyphosate degradation

Because of its effectiveness as a non-selective herbicide, along with the advent of genetically modified crops engineered for glyphosate resistance, glyphosate use has become ubiquitous on farms both large and small (Locke et al., 2015; Thierfelder et al., 2013). Additional effort should be directed towards understanding the bacterial species and pathways involved in glyphosate degradation and in developing the tools to do so. Selection is among the most powerful approaches to finding rare new activities in nature, but it requires strict controls to select against undesired activities.

Breaking the carbon-phosphorus bond is required for complete mineralization of glyphosate, and this bond contains the highest activation energy of the bonds in glyphosate (Stosiek et al., 2020). Breaking the C-P bond leaves behind the glyphosate backbone, sarcosine, which is much more likely to be fully mineralized. By contrast, if glyphosate was merely oxidized without specifically selecting for phosphorus release, then aminomethylphosphonic acid (AMPA) is produced, which still contains the carbon-phosphorus bond (Smith-Roe et al., 2023). The resulting AMPA can still serve as a source of

phosphorus, and oxidation to AMPA is sometimes a preliminary step to releasing phosphorus from glyphosate (Kamat et al., 2020). To ensure the complete mineralization of glyphosate, it must be used as a source of phosphorus. Previous attempts to examine glyphosate as a source of phosphorus, and the pathways involved, have yielded positive results. In some cases however, those results have been muddied by intentional inclusion of easily accessible phosphate in the media (W. Zhang et al., 2022). Additionally, my preliminary tests of glyphosate as a source of phosphorus produced inconsistent growth patterns including growth on phosphorus free controls, likely due to bacteria scavenging small but sufficient amounts of contaminating phosphorus. Phosphorus is an essential element and its exclusion from the media should preclude growth. Clearly, there is a need to be able to recognize when growth is due to true glyphosate degradation rather than through scavenging of contaminating phosphorus and to keep contaminating phosphorus out of the media to begin with.

2.1.2 Designing a set of best practices

Studying the degradation pathways of glyphosate presents unique challenges because of glyphosate's ability to shut down aromatic amino acid production (Pollegioni et al., 2011). Both a degradation pathway and a resistance mechanism must be present for glyphosate to act as a source of Pi. Both resistance and degradation may in theory be the same pathway if degradation is fast enough, or in some cases these pathways may be related, such as pre-processing glyphosate to AMPA before degradation by C-P lyase (Hove-Jensen et al., 2014).

C-P lyase is a relatively slow and energetically intensive pathway and is therefore unlikely to effectively impart glyphosate resistance on its own. Resistance and degradation may also be completely separate from a mechanistic standpoint, such as strains having a resistant target of glyphosate as in the case of class II EPSPS (Leino et al., 2021). Therefore, approaches must distinguish detoxification/resistance from degradation to Pi.

A good phosphorus free base media, along with a robust set of controls, is required to differentiate between: phosphorus scavengers, species that are resistant to glyphosate, and species that can both resist and degrade glyphosate. Media used in this study is based on M9 minimal salts media and lacks supplemented aromatic compounds (Miller, 1972). The aromatic amino acid producing shikimic acid pathway must therefore be active in bacteria growing in this media. Thus, bacteria who can both degrade glyphosate and resist its effects are favoured, which maximizes their potential for bioremediation applications. Buffering capacity normally provided by phosphorus salts in M9 is replaced by HEPES (4-(2-hydroethyl)-1-piperazineethanesulfonic acid).

Two positive control conditions are included, easily accessible ortho-phosphate, or ortho-phosphate along with glyphosate. The ortho-phosphate control allows us to test that our media provides all of the base elements necessary for growth of a particular strain of interest, completely separate from its interaction with glyphosate. To evaluate if strains are resistant to glyphosate separately from their ability to use it as a phosphorus source, a second positive control provides both phosphoric acid and glyphosate in the base media, under the assumption that glyphosate sensitive strains will be unable to grow under these conditions

even if they cannot degrade glyphosate to Pi. A phosphorus free negative control is also required to distinguish between organisms growing on glyphosate derived phosphorus and those growing on trace amounts of contaminating phosphorus. Though phosphorus contamination can be minimized, total elimination is virtually impossible. When testing if a bacterial strain can degrade glyphosate and use it as a sole source of phosphorus, I compared growth under four different conditions: 1.) no phosphorus source; 2.) glyphosate; 3.) glyphosate plus Pi; 4.) Pi.

Handling procedures to effectively clean phosphorus from glassware are also presented. To minimize variability between batches, a phosphorus exhaustion step for the bacteria being tested is also included. The phosphorus exhaustion step reduces variability by forcing tested bacteria to exhaust their stored phosphorus prior to testing their growth, ensuring little growth on the phosphorus free negative control.

Media and handling procedures were confirmed to result in low levels of contaminating phosphorus by the Horsman Lab of Wilfrid Laurier University, using a colourimetic assay. The results of those assays are included here.

This media was confirmed by testing the growth of *Agrobacterium pusense* RIM-Id, a suspected glyphosate degrader, and *Pseudomonas* sp. 8R6, a good phosphorus scavenger that is also glyphosate resistant. Here this media is presented, along with controls and handling procedures.

2.2 Materials and Methods

2.2.1 General

Chemicals used to make the media were purchased from Bio Basic, with the exception of; magnesium sulfate, purchased from BioShop Canada; and glyphosate, purchased from Toronto Research Chemicals. The 10-quart Instant Pot was purchased from London Drugs.

2.2.2 Base media

Media is based on M9 minimal salts media with the phosphorus buffer replaced by HEPES acid and HEPES base, and the potassium normally associated with the phosphorus buffer replaced by potassium chloride. The pH of the media was adjusted stoichiometrically using equal molar portions of HEPES free acid and HEPES sodium salt (base) to avoid phosphorus contamination by a pH probe. All glassware used was acid washed by filling with 0.1 M HCl in Milli-Q water and autoclaving for 40 minutes, high pressure setting using a 10-quart Instant Pot, this was done in an otherwise empty fume hood to contain and vent the resulting acidic steam. Glassware was then emptied and rinsed at least twice with fresh Milli-Q water and autoclaved again if sterility was necessary. Dedicated glassware was kept for each phosphorus source. Media was stored in a dark cabinet and/or in brown glass bottles to minimize glyphosate degradation by UV light. When acid washed glass was not used, then single use plastics were used instead.

Media was made in two parts, an autoclaved salts base solution at 1.1x strength (900 mL are autoclaved for every 1000 mL intended final volume) and a 10x (100 mL for every

1000 mL final volume) filter sterilized portion. With the exception of the trace elements solution, the chemicals used are from stocks dedicated to making phosphorus free media to avoid contamination. The autoclaved portion contains: NaCl, 10 g; $MgSO_4*7H_2O$, 0.5 g; $CaCl_2$, 0.015 g (added as 1 mL of 1000x 15 g/L stock); KCl, 1.6 g; NH_4Cl, 1 g; and 900 mL Milli-Q water. The 10x filter sterilized portion is made as follows: HEPES free acid, 11.9 g (0.5 mol); HEPES-Na (base), 13.0 g (0.5 mol); trace elements, 1 mL; D-glucose, 3 g; phosphorus source (glyphosate, NaH_2PO_4, or none), variable amounts; Milli-Q to 100 mL. The trace element solution is as follows: NaEDTA, 1 g; K_2SO_4, 1 g; H_3BO_3, 0.1 g; $ZnSO_4*7H_2O$, 0.1 g; $CuSO_4*7H_2O$, 0.05 g; $MnCl_2*4H_2O$, 0.05 g; $Na_2MoO_4*2H_2O$, 0.1 g; $CoSO_4$, 0.1 g; Milli-Q to 1000 mL. The trace element solution was autoclaved and stored in the fridge. When making the filter sterilized portion, disposable 50 mL conical vials were used to avoid cross contamination of the different phosphorus sources. The pH of the final media is approximately 7.2. To help the glyphosate dissolve, the dissolved buffer portion is added to the dry glyphosate before it is filter sterilized. Glyphosate does not dissolve well in unbuffered milli-Q water. When making multiple medias with different phosphorus sources large base and buffer solutions are made and each phosphorus source is added individually. This allows greater consistency across phosphorus sources when comparing growth between multiple phosphorus sources. The buffer solution was mixed with the phosphorus source in single use plastic conical tubes to keep glassware phosphorus free. When filter sterilizing, a different and dedicated syringe for each phosphorus source was used. When making agar plates, 15 g of Noble agar is added to the larger autoclaved portion and all other dry chemicals are instead added to the smaller filter sterilized portion. Noble agar solidifies most

consistently when it is autoclaved with Milli-Q alone and the other components added after autoclaving. Recipe tables are given in appendix A.

2.2.3 Testing phosphorus contamination levels

In order to test if our media is sufficiently free of phosphorus, collaborators Afshan Ardalan, and Minghui Ma of the Horsman Lab at Wilfrid Laurier University performed a colourimetric assay to assess phosphorus contamination, using an ascorbic acid two reagent test, method 365.3 from the United States Environmental Protection Agency (United States Environmental Protection Agency, 1978).

2.2.4 Testing isolated strains for glyphosate degradation

To verify this media, isolated strains were tested for glyphosate degradation, glyphosate resistance, and glyphosate scavenging. Strains that had previously given inconsistent results were *Agrobacterium pusense* RIM-Id, and *Pseudomonas* sp. 8R6. These were originally isolated for unrelated studies. Both strains were streaked from -70°C onto tryptic soy agar, and grown at 30° C. A single colony from each of these was selected and inoculated into 5 mL of tryptic soy broth and grown with shaking. 1 mL of each culture was spun down at 10,000 rcf for 2 minutes, decanted, and resuspended in 1 mL of media without any source of added phosphorus. 100 µL of the cleaned culture was inoculated into 5 mL of media without any source of phosphorus and grown overnight at 30°C with shaking to exhaust any stored

phosphorus. 1000 µL of the phosphorus-exhausted culture was again pelleted, decanted, and resuspended in 200 µL of phosphorus free media. 3 µL of this 5x phosphorus exhausted culture was inoculated into 297 µL of media a 100 well honeycomb microplate for use in a Bioscreen C growth curve plate reader. The negative control received 3 µL of media without added phosphorus. Each culture or control was grown in duplicate in the microplate. The media had one of 4 phosphorus conditions: no phosphorus added; 0.71 g/L (0.006M) NaH_2PO_4; 1 g/L (0.006M) glyphosate & 0.71 g/L (0.006M) NaH_2PO_4; or 1 g/L glyphosate (0.006M). These were grown in duplicate using a BioscreenC growth curve machine at 30° C with continuous shaking, and readings taken every 15 minutes. These conditions served to check that the media was sufficiently free of phosphorus to preclude significant growth (phosphorus free treatment), to check if the media provided all of the nutrients required for growth (ortho-phosphate, NaH_2PO_4), to check if the bacteria was sensitive to glyphosate (glyphosate and ortho-phosphate), and finally to check if the bacteria was able to use glyphosate as its sole source of phosphorus.

Duplicate readings were zeroed, averaged, and graphed. Error bars show the highest and lowest measurement at that time point.

2.3 Results

2.3.1 Phosphorus contamination and growth results

Extremely low levels of phosphorus contamination were found in the base media (without any source of added phosphorus). The liquid media was found to contain 0.78 mg/L of phosphorus (Pi) with a standard deviation of +/- 0.03 mg/L. When adding Noble agar for solidified media, this level rose to approximately 1.75 mg/L Pi. This low level of phosphorus contamination is a significant improvement over previous versions of this media which contained enough contaminating phosphorus to allow growth even on the intended phosphorus free control.

With these practices to reduce phosphorus contamination, true glyphosate degraders can be differentiated from glyphosate tolerant phosphorus scavengers. In the case of the *Agrobacterium pusense* RIM-Id, it grows well on glyphosate as sole source of phosphorus, but does not grow well in the phosphorus free control. *A. pusense* RIM-Id is therefore a glyphosate degrading strain and growth is not due to phosphors scavenging. *Pseudomonas* sp. 8R6 is glyphosate tolerant and will grow in the presence of glyphosate, but does not degrade glyphosate to usable Pi (Figure 2.1). Using previous iterations of this media, inconsistent results were obtained with many of the tested strains, including *Pseudomonas* sp. 8R6. These inconsistencies led to the erroneous conclusion that it was degrading glyphosate.

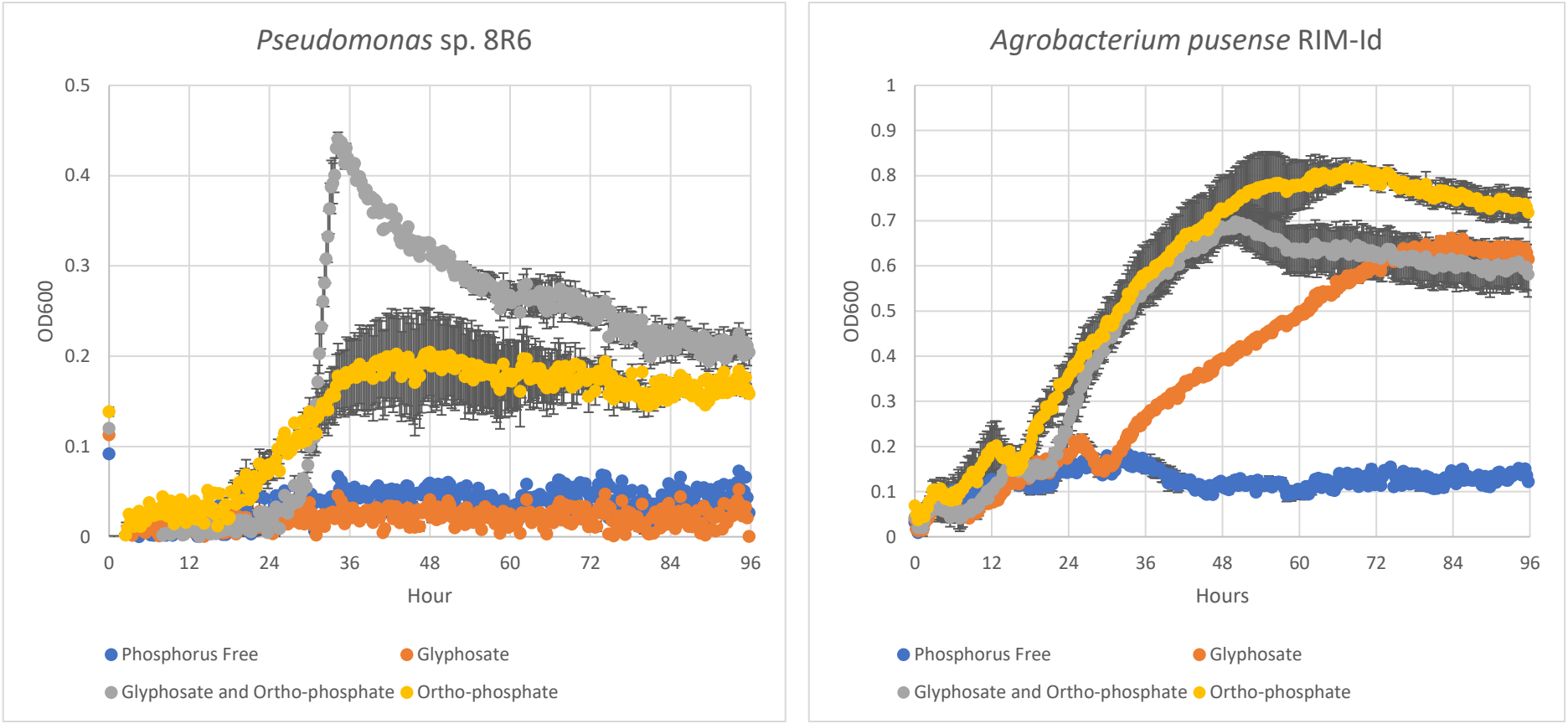


Figure 2.1 Growth Results: Growth of *Pseudomonas* sp. 8R6 and *Agrobacterium pusense* RIM-Id on glyphosate. *Pseudomonas* sp. 8R6 is resistant to glyphosate, and able to grow in its presence as long as an additional phosphorus source is provided. By contrast, it cannot grow on glyphosate as a sole source of phosphorus. *Agrobacterium pusense* sp. RIM-Id is both able to survive glyphosate and grow well on the base media, it is also able to grow well on glyphosate as a sole source of phosphorus. Additionally, little growth is seen when no phosphorus is provided, showing that our media is sufficiently free of phosphorus to prevent effective phosphorus scavenging. Error bars show the highest and lowest measurements, points show the average. Note that *Pseudomonas* sp. 8R6 showed approximately half the maximum growth of *Agrobacterium pusense* RIM-Id and the Y-axis is scaled accordingly.

2.4 Discussion

2.4.1 Minimizing phosphorus contamination

Many bacterial strains can sustain growth on small amounts of contaminating phosphorus. However, glyphosate degradation and phosphorus scavenging can be differentiated with proper controls and careful attention to minimize phosphorus contamination. Colourimetric analysis of ortho-phosphate determined very little phosphorus contamination in the liquid media (0.78 mg/L of phosphorus). However, adding 15 g/L Noble Agar increased the phosphorus contamination level to 1.75 mg/L. While this is still a very small amount of contamination, it is prudent to double check isolates selected on Noble Agar plates in liquid media to confirm that they show greater growth in the presence of glyphosate than in the absence of any added phosphorus.

Previous attempts to test glyphosate as a source of phosphorus produced inconsistent growth patterns and necessitated a set of standard practices to minimize contamination. To minimize contamination; it is important to use Milli-Q water rather than deionized water, to include acid washing step for glassware, to pH the media stoichiometrically rather than trichromatically as a pH probe itself can be a source of phosphorus contamination (including from carryover from the calibration buffers), and to include a preliminary step to grow strains of interest in a phosphorus free media to exhaust them of stored phosphorus (which may have been stored from the previous media the strains were grown on). Before implementing these controls, good glyphosate degrading organisms could not be effectively differentiated from organisms that are good at scavenging or storing phosphorus, leading to frequent false positives. These steps help to prevent false positives (growth that is not depended on

glyphosate degradation), including a phosphorus free negative control confirms that these steps sufficiently prevent phosphorus contamination, and show that growth on glyphosate as the sole source of phosphorus is truly due to degradation of the glyphosate itself.

While other studies have examined glyphosate mineralization and have recognized the need to avoid AMPA accumulation, they have not always used a totally phosphorus free base media or pure glyphosate. For example, Zhang et al 2022 reports complete mineralization of glyphosate however they do so using a media that contains intentionally added phosphoric acid (W. Zhang et al., 2022). In their study, they add the equivalent of 1.44 g/L of PO_4 as Na_2HPO_4*12 H_2O and KH_2PO_4, while their glyphosate supplementation (up to 0.8 g/L) provides a maximum equivalent of 0.45 g/L PO_4. Clearly, not all glyphosate degradation studies are performed using truly phosphorus free media.

In addition to being sure to reduce outside sources of phosphorus contamination, it is also important to use purified glyphosate rather than agricultural or consumer grade herbicides that contain glyphosate (such as Monsanto's Roundup WeatherMAX, Touchdown Total, Durango DMA and many others). Frequently, these herbicides contain surfactants including polyoxyethylene tallow amine, and while it is important to study the effects of these chemicals on the microbiota in agriculture and the environment, their inclusion would obscure the effect of glyphosate on the shikimate pathway as well as on the degradation pathways by inhibiting bacteria in a way that is not specific to glyphosate or the shikimate pathway (Tush et al., 2013). It has been shown that polyoxyethylene tallow amine can inhibit *Vibrio fischeri* growth at doses as low as 10.2 mg/L (Tush et al., 2013). This effect is due to

destabilizing cell membranes, rather than pathway inhibition. In addition, agricultural and consumer grade glyphosate formulations are unlikely to have the stringent purity standards that lab grade glyphosate has, and therefore would be expected to contain some contaminating and accessible Pi. It is very common for studies to examine the molecular aspects of glyphosate mineralization using herbicide grade mixtures intended for agriculture. These mixtures include surfactants to help the glyphosate enter the plant, as well as other components for shelf stability and operator convenience. Once such study was published by González-Valenzuela and Dussán in 2018 in which they studied the ability of *Lysinibacillus sphaericus* to degrade glyphosate through the sarcosine pathway, however their media used agricultural Roundup rather than pure glyphosate (González-Valenzuela & Dussán, 2018).

2.4.2 Tested strains

Previous iterations of this media have had trace amounts of phosphorus at varying and inconsistent concentrations. This has led to inconsistent results when screening strains for their ability to grow on glyphosate as sole phosphorus source. Among them, the *Pseudomonas* strain 8R6 has at times appeared to degrade glyphosate, and at times not. Further testing (Figure 2.1) suggests that it cannot degrade glyphosate as a sole source of phosphorus, but may be a good phosphorus scavenger that is resistant to glyphosate. The wide variability in phosphorus requirements and phosphorus scavenging ability by bacteria necessitates a phosphorus free control, along with a phosphorus exhaustion step, to be included in any test of glyphosate as a source of phosphorus. Additionally, testing for growth

in the presence of glyphosate but not necessarily on glyphosate as a phosphorus source, provides essential information when evaluating the glyphosate degradation ability of strains of interest. Knowing if the inclusion of glyphosate has an inhibitory effect on the bacterial growth can elucidate the differences between glyphosate degradation and glyphosate resistance. A spike in absorbance was seen when *Pseudomonas* sp. 8R6 was given both glyphosate and ortho-phosphate, but not when ortho-phosphate was provided alone. It may be that glyphosate is being used as a carbon source but not as a phosphorus source, by being first oxidized into AMPA and glyoxylate by glycine oxidase, followed by consumption of glyoxylate as an additional source of carbon (Flynn et al., 2017; Pollegioni et al., 2011). This may represent a positive effect of glyphosate on the growth of *Pseudomonas* sp. 8R6, and serves to further highlight the importance of testing growth on glyphosate along with ortho-phosphate rather than only testing them separately.

In contrast, *Agrobacterium pusense* RIM-Id does appear to be a good glyphosate degrader, and is glyphosate resistant (Figure 2.1), in line with other *Agrobacterium* species (Green & Owen, 2011; Hove-Jensen et al., 2014). Clearly the mechanisms used by *Agrobacterium pusense* RIM-Id to degrade glyphosate are worthy of further study (Chapter 4).

2.5 Conclusions

Glyphosate as the sole source of phosphorus is a useful selection criteria when attempting to find glyphosate degrading organisms. When creating media to select for this, the base media

must be sufficiently and consistently free of phosphorus to allow effective selection. This media achieves this through careful handling, pure and dedicated chemicals, and stoichiometric rather than titrimetric pHing. When testing isolated strains for glyphosate degradation as a source of phosphorus, it is important to include a set of controls to test for phosphorus scavenging (no phosphorus added), glyphosate resistance (glyphosate along with ortho-phosphate), and ability to grow on the base media (ortho-phosphate).

Chapter 3

Enrichment Culture

3.1 Introduction

3.1.1 Glyphosate's effect on soil and community microbiology

Because of its ability to shut down the aromatic amino acid production pathway in bacteria, and its potential as a source of phosphorus, the effects of glyphosate on soil microbiology can be diverse and complex (Hove-Jensen et al., 2014; Pollegioni et al., 2011). Attempts to study these effects often contradict each other, with some studies showing negative effects of glyphosate on soil and environmental health, and others showing benign or even positive effects (Arango et al., 2014; Banks et al., 2014; Druille et al., 2015; Kepler et al., 2020). Glyphosate has the potential to select against glyphosate sensitive bacteria if they cannot find a source of aromatic compounds other than anabolism, while strains that survive may simply resist it, or may be able to make use of it as a source of phosphorus, carbon, and/or nitrogen (Hove-Jensen et al., 2014; Pollegioni et al., 2011).

The incredible bacterial diversity of healthy agricultural soil provides researchers a source of novel strains and diverse biochemistries. However, simplifying these communities down to a core set of species both able to grow in the laboratory and capable of performing a function of interest remains a challenge. A well-designed enrichment culture, with conditions chosen to select for chemistry and strains of interest is how this challenge can be solved. Additionally, enrichment cultures select for species able to perform a chemistry of interest

(such as carbon or phosphorus metabolism) and for strains able to grow under unintentional selective pressures inherent to growth in the laboratory. Both selection for the condition of interest (glyphosate degradation), and selection for growth on defined medium, are different qualities worthy of analysis, especially if we want to apply these lessons to our understanding of natural systems.

Previous enrichments using glyphosate as the sole source of phosphorus have been performed. While those studies have provided valuable insight into our understanding of which species can degrade glyphosate, they did not compare selection by different phosphorus sources in the same base media, such as easily accessible ortho-phosphate versus glyphosate (Ibrahim et al., 2023; Massot et al., 2021). This leaves a blind spot where we are unable to separate growth on the media and laboratory conditions generally from selection for glyphosate degradation specifically. By testing enrichment with multiple phosphorus sources, I can examine which species are selected by glyphosate but not strongly selected by ortho-phosphate.

Valuable insights about the role of conditions for selection can be gained by examining the communities themselves, how they change over time, and how they differ from each other. Specifically, using the phosphorus free media introduced in Chapter 2 with either ortho-phosphate (NaH_2PO4) or glyphosate as the sole source of phosphorus, species from soil from a corn or soy field previously exposed to glyphosate will be selected for growth on either ortho-phosphate or glyphosate using enrichment cultures. Additionally,

these glyphosate degrading communities serve as a source of glyphosate degrading bacteria for further analysis, presented in Chapter 4.

3.1.2 Diversity analysis

Measures of alpha-diversity, the diversity within a sample, can give us an understanding of how the community simplifies over its period of enrichment. It is expected that moving a diverse soil microbiota into defined media in the laboratory should greatly decrease common measures of diversity, and that diversity should continue to decrease as the enrichment continues (Jiao et al., 2016). Diversity can be measured as the total number of species found in a community, simply reported as species richness, or can include a component that examines the evenness of that community (Shannon's diversity) (Chao & Shen, 2003; Willis, 2019). In a high evenness community, the community mostly contains species each with a similar population size. In contrast, a low evenness community may have an equal number of species present, but be dominated by a single or a few particularly dominant species (Chao & Shen, 2003; Willis, 2019).

By contrast, beta-diversity looks at the difference between samples and is extremely helpful when comparing how communities differ due to different selective conditions. There is inherent difficulty however when comparing multiple communities that differ over multiple dimensions, with differences in many species rather than just differences of a few species. Finding which communities are most similar and most divergent from each, and if those differences align with growth conditions (such as phosphorus source) requires a method to compute those differences and to plot them. Differences are calculated using

diversity measures such as Bray-Curtis, which looks at the differences between samples, and weighted UniFrac, which adds an additional component that takes into account the phylogenetic relationship between the species present in the samples (Lozupone & Knight, 2005; Su, 2021). Examining both Bray-Curtis, and weighted UniFrac allows both a measure of the absolute differences between samples, as well as the phylogenetically relevant differences. Differences between cultures are plotted using principal coordinate analysis.

3.1.3 Aims

Using the media validated in Chapter 2, enrichment cultures are created to examine glyphosate degrading bacteria from a corn (*Zea mays*) and a soy (*Glycine max*) field, in Southwestern Ontario between Waterloo and Guelph. Enrichments are made to compare species selected for by glyphosate as sole phosphorus source, to species able to grow on the media with easily accessible ortho-phosphate (as NaH_2PO_4) as phosphorus source. The fourth hypervariable (V4) region of the 16S rRNA gene of the community members is sequenced for each of the subcultures. Specific species dominating each subculture are analyzed in order to elucidate the effect of glyphosate on bacterial communities. Comparison within the enrichments is done by calculating alpha diversity with species richness, and Shannon's diversity. Beta diversity is calculated and compared using Bray-Curtis as well as weighted UniFrac. For the glyphosate degrading enrichment cultures, degradation is quantified using an established commercial ELISA kit in order to examine the glyphosate degrading potential of these communities, and potential applications in glyphosate bioremediation.

3.2 Materials and Methods

3.2.1 General materials

The ABRAXIS glyphosate ELISA and derivatization kit used to quantify glyphosate was purchased from Gold Standard Diagnostics Horsham, of Horsham, PA, USA. Chemicals used to make the media were purchased from Bio Basic, with the exception of; magnesium sulfate, purchased from BioShop Canada; and glyphosate, purchased from Toronto Research Chemicals. A full chemical list is provided in Chapter 2.

3.2.2 Enrichment culture

Using the previously validated media (Chapter 2), a series of enrichment cultures were set up using two sources of inoculant, soil from a corn (*Zea mays*) field or soil from a soy (*Glycine max*) field, which had been in a long term corn-soy rotation. Fields were within 1 km of each other and were located in Southwestern Ontario, between Waterloo and Guelph and contained Guelph loam soil. Soil was collected in August of 2020. Both the corn and the soy field had a history of glyphosate exposure 1-2 times per year going back at least 5 years. Soil was stored in the fridge at 4°C for 2 months before use. 1 g of soil was placed in 50 mL of phosphorus free media, vortexed for 5 minutes and heavy solids (sand) was allowed to settle for 2 minutes. 10 mL was poured off and pelleted at 7000 rcf for 5 minutes, the pellet was resuspended in 10 mL fresh defined phosphorus media without phosphorus. This served to help wash soluble minerals out of the bacterial mixture. This was done once for each of the

soil sources. 100 µL of this soil solution was used as inoculant. The enrichment therefore had one of two soil sources, as well as one of two sources of phosphorus, 1 g/L (0.006M) glyphosate or 0.71 g/L (0.006 M) NaH_2PO_4. Therefore 4 treatment conditions were used: glyphosate and soy field soil, ortho-phosphate and soy field soil, glyphosate and corn field soil, ortho-phosphate and corn field soil. Each treatment was done in triplicate, for a total of 12 separate lines. These lines were given arbitrary letters to designate them as separate (some letters were skipped if they were too similar to another letter or number, for example O). Enrichment cultures are labeled first with the phosphorus source (G for glyphosate, or P for ortho-phosphate), followed by the soil source (S for soil from a soy, C for soil from a corn), then the arbitrary series name and the enrichment number in that series (A1 was used to inoculate A2). E.g GS-A2 is the second subculture in series A, it is always grown on glyphosate and it was started using soil from a soy field. Each subculture was continued for a total of 6 generations, giving 36 glyphosate subcultures and 36 ortho-phosphate subcultures.

Enrichment was done using acid washed (phosphorus free) 16x150 mm test tubes (Fisher Scientific), using 5 mL of the respective media. Cultures were grown at 30°C with shaking at 180 rpm, in sterile glass culture tubes were held at approximately a 50° angle (in an 18 mm tube holder). Twice weekly (Monday and Thursday), 100 µL of culture was taken from each tube and used to start a new subculture. At this point, 750 µL portions of each of the enrichment cultures were diluted 1:2 with 14% DMSO in phosphorus free media and saved at -70°C for future sequencing, future growth tests (Section 3.2.5), and so that individual strains could be isolated (Chapter 4).

As a control, a phosphorus free culture was created from each of the glyphosate subcultures. This control was to confirm that there would be little growth if a source of phosphorus was not included to show that we were not isolating phosphorus scavengers rather than glyphosate degraders. Phosphorus free cultures were checked visually to ensure that they experienced little growth compared to the corresponding glyphosate subculture.

3.2.3 DNA isolation, sequencing, and species identification

DNA from the saved samples was isolated using the Metagenom Bio Life Science Inc. Sox Soil Kit (Waterloo, Ontario). Despite the kit being designed to extract DNA from bulk soil, it has been found to perform well when isolating DNA from pelleted liquid cultures as well. A 750 µL portion of original enrichment was thawed and pelleted by spinning for 5 minutes at 10,000 rcf. Supernatant was decanted and the pellet resuspended in Sox homogenization buffer. DNA isolation proceeded following the manufacturers instructions. Genomic DNA was eluted from the column using sterile, DNAse free water.

Once the DNA was isolated, sequencing was conducted by Metagenome Bio Life Science Inc, Waterloo Ontario. The V4 region of the 16S rRNA gene was then amplified in triplicate using Universal 16S V4 rRNA primers 515FB (5'-GTGYCAGCMGCCGCGGTAA) and 806RB (5'- GGACTACNVGGGTWTCTAAT) on an Illumina MiSeq (Walters et al., 2015). The triplicate PCR products were then pooled for each sample and resolved on 2% TAE agarose gel. Equal amounts of PCR product amplicons were pooled, gel purified, and quantified using Qubit dsDNA HS Assay Kit (Thermo Fisher

Scientific Inc.). Library DNA was sequenced with Miseq Reagent Kit v2 (2x250 cycles), generating FASTQ files for taxonomic and sequence analysis. Demultiplexed sequences were processed using cutadapt (primer removal) and DADA2 v. 1.22 (sample interference) (Callahan et al., 2016; Martin, 2011). Reads were truncated at decreasing quality scores and assembled.

3.2.4 Community analysis

After quality filtering (chimera, organelles) and taxonomic assignment, an abundance table was constructed. This table records the number of sequence reads for each amplified sequence variant for each sample. Taxonomy was assigned to representative sequences using IdTaxa from the DECIPHER package (Murali et al., 2018). The package was trained against reference database SILVA release 138 at 99% to create an abundance table of reads in each sample (Quast et al., 2012). Actual sequence variants (ASV) were used in order to avoid merging distinct reads that could represent distinct species. After taxonomy assignment, samples with less than 2000 total reads were re-sequenced and taxonomy was again assigned. These tables were then merged.

The abundance table was manually annotated so that the lowest level of identified phylogeny was repeated to fill in the unknown levels of phylogeny, and a unique species identifier was added (sp.1, sp.2, etc) so that distinct reads would not be merged at the species level and so that distinct but unidentified reads would not be dropped. Additionally, the representative sequence for strains of interest were identified via BLAST and manually

annotated by highest BLAST hit that had a full species name, as long as the hit was over 95% identical. DECIPHER did not properly identify the *Agrobacterium* genus from other Rhizobiaceae, instead it calling multiple distinct genera of Rhizobiaceae *Allorhizobium-Neorhizobium-Pararhizobium* because of frequent reorganization within the Rhizobiaceae (Mousavi et al., 2014, 2015). *Allorhizobium-Neorhizobium-Pararhizobium* in particular were therefore manually annotated as they were a large portion of the library and of particular interest for further study. This now merged and annotated table was graphed using ggplot2 running in RStudio (RStudio Team, 2020; Wickham, 2016). The abundance table was converted to relative abundance, and then graphed as bubble plots showing abundance at various taxonomic hierarchy. Groups (species, genera, families, etc.) that were below 5% relative abundance were sorted into 'Other'.

Diversity analysis was performed using phyloseq, and DECIPHER & ape were used to create a phylogenetic tree for UniFrac ordination (McMurdie & Holmes, 2013; Paradis & Schliep, 2019; Wright, 2016). Diversity plots were displayed using ggplot2 in RStudio (RStudio Team, 2020; Wickham, 2016).

3.2.5 Quantifying enrichment culture growth on glyphosate

After the enrichment series was performed, glyphosate enrichments were regrown to quantify both their growth on glyphosate as the sole source of phosphorus, and to quantify the amount of glyphosate they degraded. To test whether growth and glyphosate degradation improved over the course of the enrichment, enrichments 1 and 6 were tested for growth on glyphosate

by optical density using a BioscreenC growth curve machine, reading at 600 nm every hour for 96 hours. End point glyphosate was quantified using the ABRAXIS glyphosate ELISA and derivatization 96 well test kit, from Gold Standard Diagnostics Horsham.

Previously saved samples of enrichments 1 and 6 (750 µL of enrichment in 750 µL of 14% DMSO in phosphorus free media, stored at -70°C) were thawed on ice, and 200 µL was used to inoculate 5 mL of defined phosphorus media without any phosphorus. This was grown overnight at 30 ℃ with shaking to exhaust stored phosphorus. 30 µL of the phosphorus exhausted culture was used to inoculate 270 µL of base media that was either phosphorus free or contained 0.025 g/L glyphosate. 0.025 g/L was selected because this was the point at which phosphorus became limiting for *Agrobacterium pusense* RIM-Id (Chapter 4.3.7). Previous tests showed that when using 1 g/L glyphosate, too small a portion of the initial glyphosate was degraded to show a difference between initial glyphosate and final glyphosate. Growth on each media was tested in triplicate and grown on the BioscreenC growth curve machine at 30 ℃ with shaking for 96 hours. Optical density readings were taken every hour for 96 hours.

After 96 hours, the cultures were taken out of the BioscreenC and stored at -20 ℃ for future glyphosate quantification. Glyphosate was quantified using a commercial ELISA glyphosate test kit (Gold Standard Diagnostics Horsham, Inc). The kit was used according to the manufacturer's specifications. Samples were diluted at 10^{-4} using 1/10 serial dilutions to bring samples within the quantification range specified by the kit (0 to 4 ppb (or ng/mL)). Dilution was done using milli-Q water, on ice, with 15 sections of vortexing on maximum

speed between each dilution. A standard curve was created using the initial defined phosphorus media with 0.025 g/L glyphosate, diluted with phosphorus free media. The standard curve was created at 0, 0.00625, 0.0125, 0.01875, and 0.025 g/L glyphosate, then diluted 10^{-4} using the same procedure as above.

Glyphosate was quantified according to the manufacturer's directions, by dividing the absorbance at that glyphosate concentration by the absorbance at 0 glyphosate. This absorbance ratio was plotted against glyphosate concentration using excel on a logarithmic X-axis. The line of best fit was found and used to calculate the concentration of glyphosate remaining in each of the cultures containing glyphosate as the sole source of phosphorus.

3.3 Results

3.3.1 Enrichment community

The abundance tables were converted to relative abundance and graphed. Graphs were split by inoculant (soil from either a corn or soy field) and both amendments (glyphosate or ortho-phosphate) are shown on the same graph. Subculture series are kept together and shown in order of subculture number from the first to the sixth subculture.

Similar enrichment profiles were found independent of inoculant source. Glyphosate was found to enrich for *Ochrobactrum rhizosphaerae*, *Agrobacterium tumefaciens*, and *Ensifer adherens* (Figures 3.1 and 3.2). Additionally, multiple species within *Ochrobactrum*, *Agrobacterium*, and *Ensifer* genera were found to be enriched, making the effect stronger at

the genus level (Figures 3.3 and 3.4). These species are within the family Rhizobiaceae, which dominates the glyphosate enrichments if sorting relative abundance at the family level of phylogeny (Figures 3.5 and 3.6).

When ortho-phosphate was used as the source of phosphorus, a single unidentified species of *Klebsiella* came to dominate the enrichments at the species level, along with more minor contributions by multiple *Pseudomonas* species (Figures 3.1 and 3.2). At the family level, Enterobacteraceae (of which *Klebsiella* is a member) and Pseudomonadaceae dominate the ortho-phosphate enrichments (Figures 3.5 and 3.6).

These differences between glyphosate or ortho-phosphate as a source of phosphorus persist all the way through the order (Figures 3.7 and 3.8) and to the class level (Figures 3.9 and 3.10). At the class level, glyphosate subcultures are dominated by Alphaproteobacteria and ortho-phosphate subcultures dominated by Gammaproteobacteria (Figures 3.9 and 3.10).

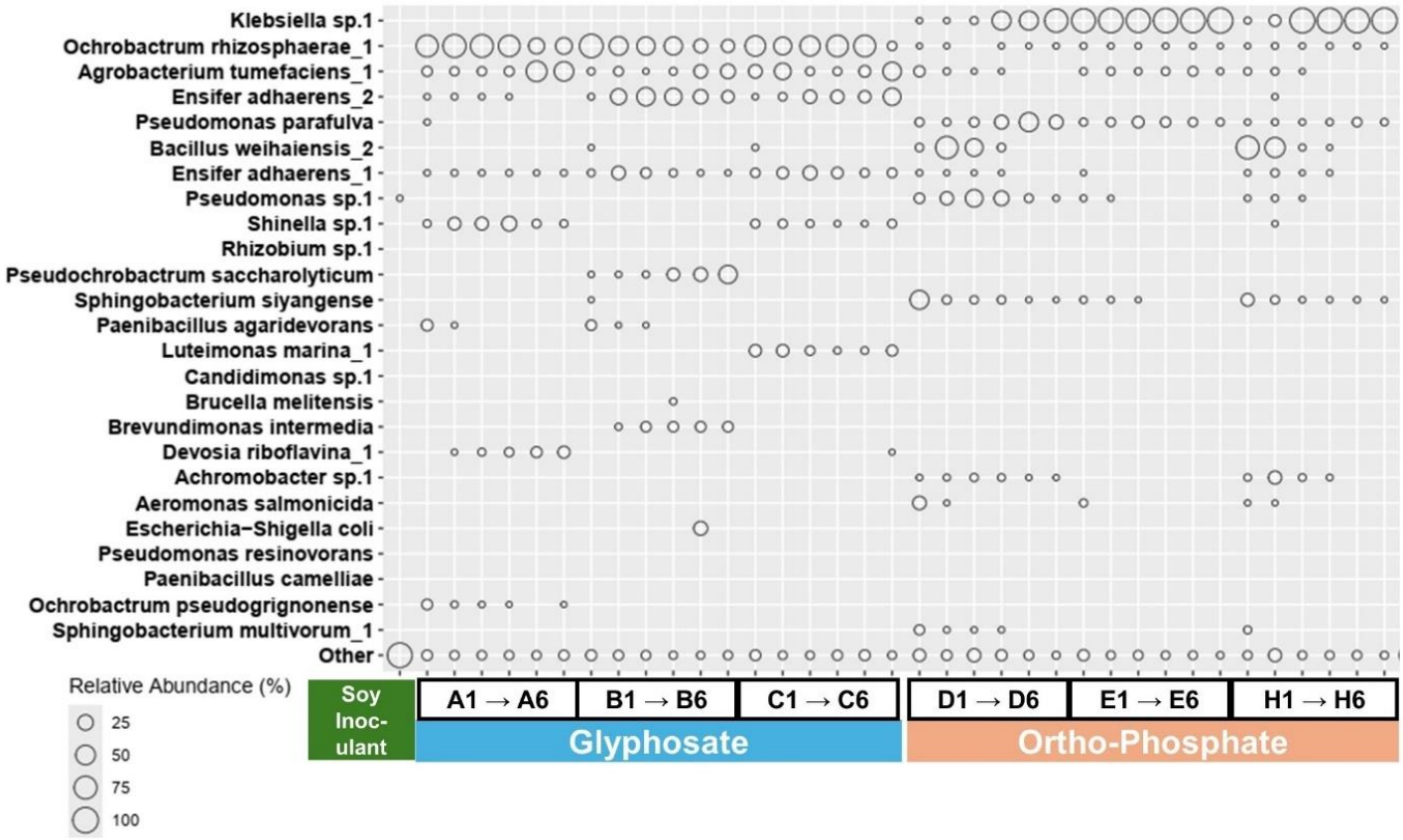


Figure 3.1 Relative Species Abundance of Soy Subcultures: Relative abundance of species present at over 5% is shown. Samples were inoculated with soil from a soy field and enriched on either glyphosate (subculture A, B, and C) or ortho-phosphate (subcultures D, E, and H). Subcultures are shown in order of creation within their series, from subculture 1 to subculture 6. The original inoculant is also shown. Each ASV was given a unique species identity, so that distinct ASVs would not be merged. Glyphosate subcultures show clear enrichment for *Ochrobactrum rhizosphaerae*, *Agrobacterium tumefaciens*, and some enrichment for *Ensifer adherens*. Though *O. rhizosphaerae* is initially enriched, it appears to lose some of its abundance by subculture 6. In contrast, subcultures selected on ortho-phosphate show significant enrichment for a species of *Klebsiella*, which quickly comes to dominate the culture, along with more minor contributions by multiple *Pseudomonas* species.

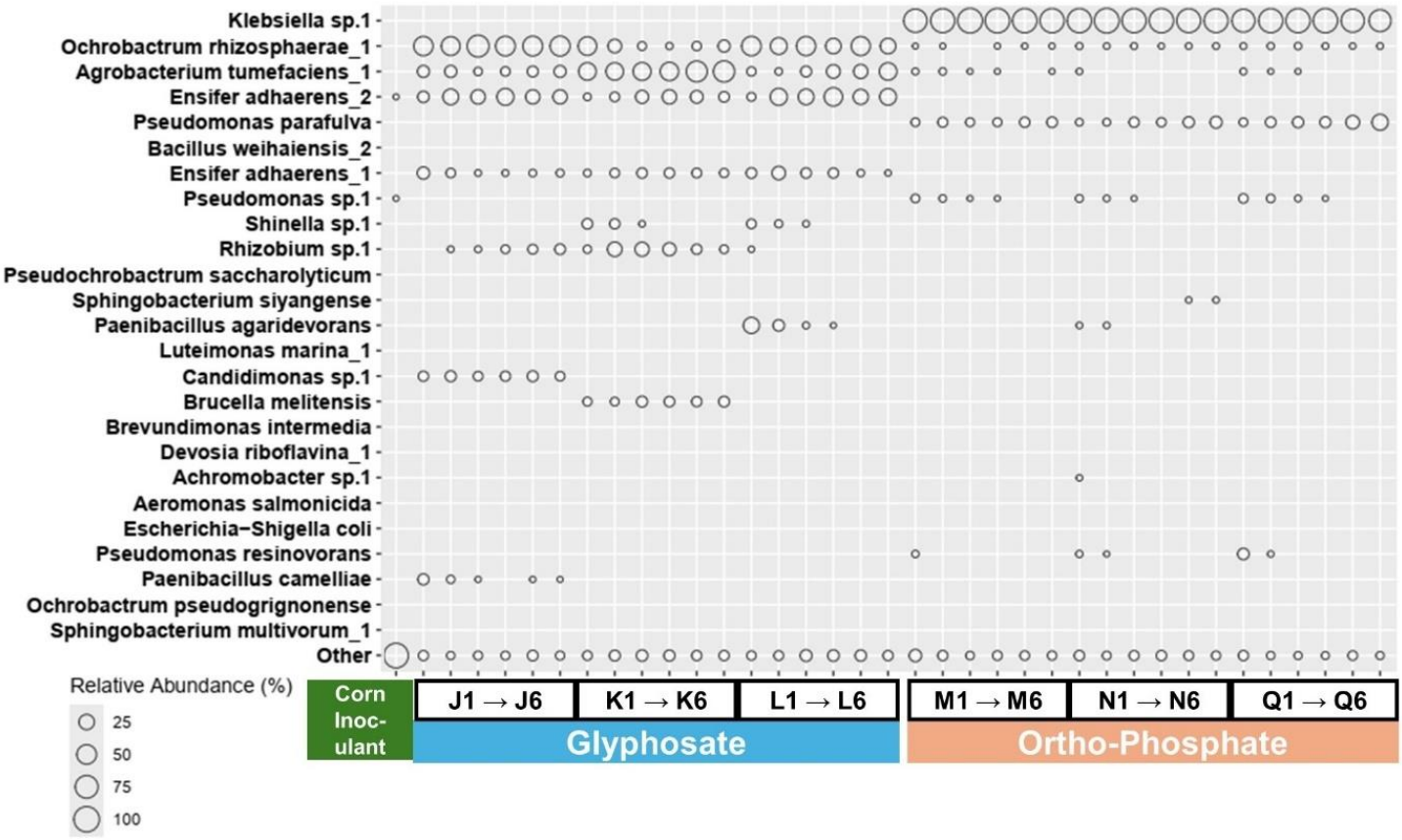


Figure 3.2 Relative Species Abundance of Corn Subcultures: Relative abundance of species present at over 5% is shown. Samples were inoculated with soil from a soy field and enriched on either glyphosate (subculture A, B, and C) or ortho-phosphate (subcultures D, E, and H). Subcultures are shown in order of creation within their series, from subculture 1 to subculture 6. The original inoculant is also shown. Each ASV was given a unique species identity, so that distinct ASVs would not be merged. Glyphosate subcultures show clear enrichment for *Ochrobactrum rhizosphaerae*, *Agrobacterium tumefaciens*, and some enrichment for *Ensifer adherens*. Though *O. rhizosphaerae* is initially enriched, it appears to lose some of its abundance by subculture 6. In contrast, subcultures selected on ortho-phosphate show significant enrichment for a species of *Klebsiella*, which immediately comes to dominate the culture, along with more minor contributions by multiple *Pseudomonas* species.

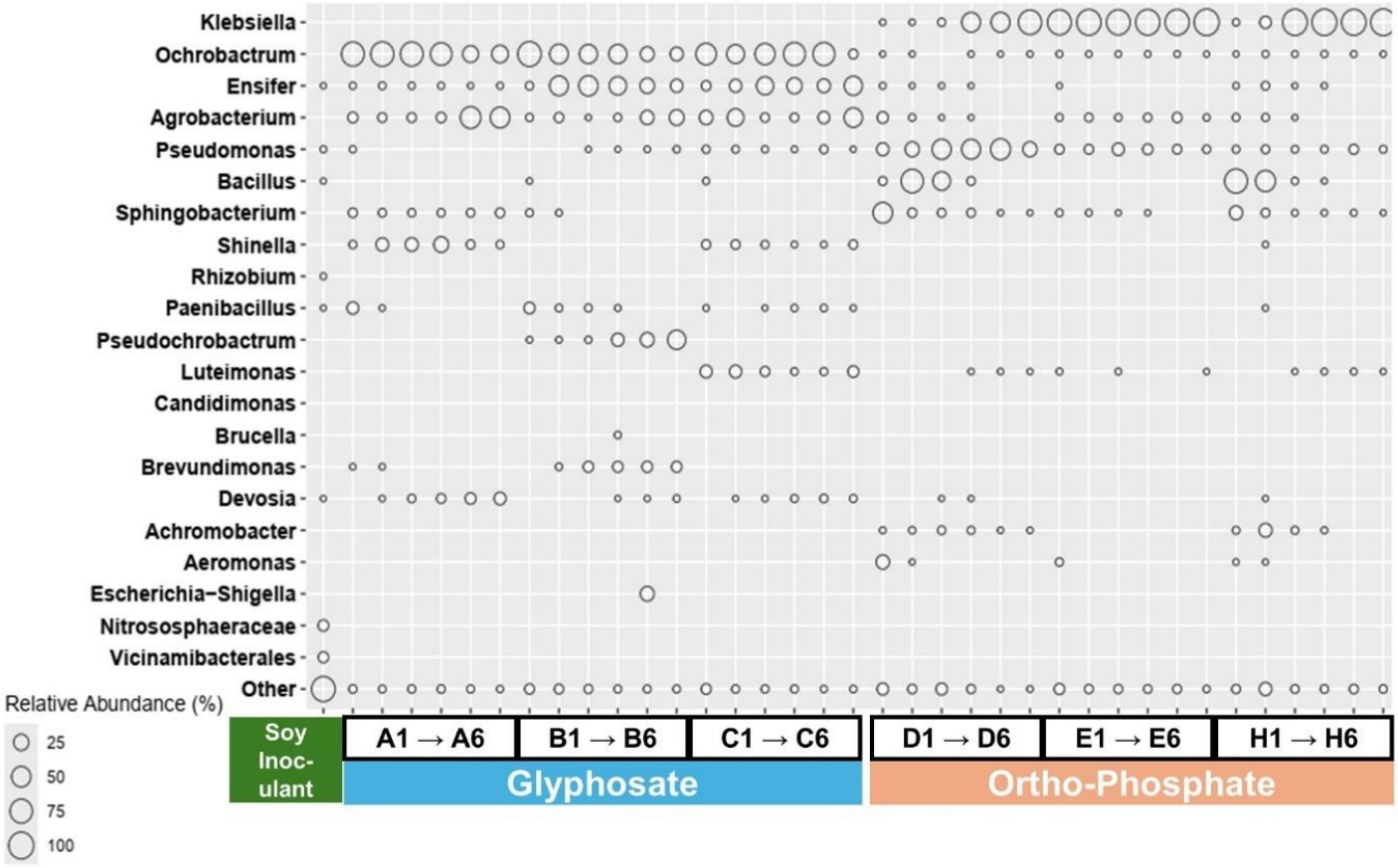


Figure 3.3 Relative Genus Abundance of Soy Subcultures: Binning reads by genus again shows enrichment by glyphosate for *Ochrobactrum*, *Agrobacterium*, and *Ensifer*, and enrichment by ortho-phosphate for *Klebsiella* and *Pseudomonas*.

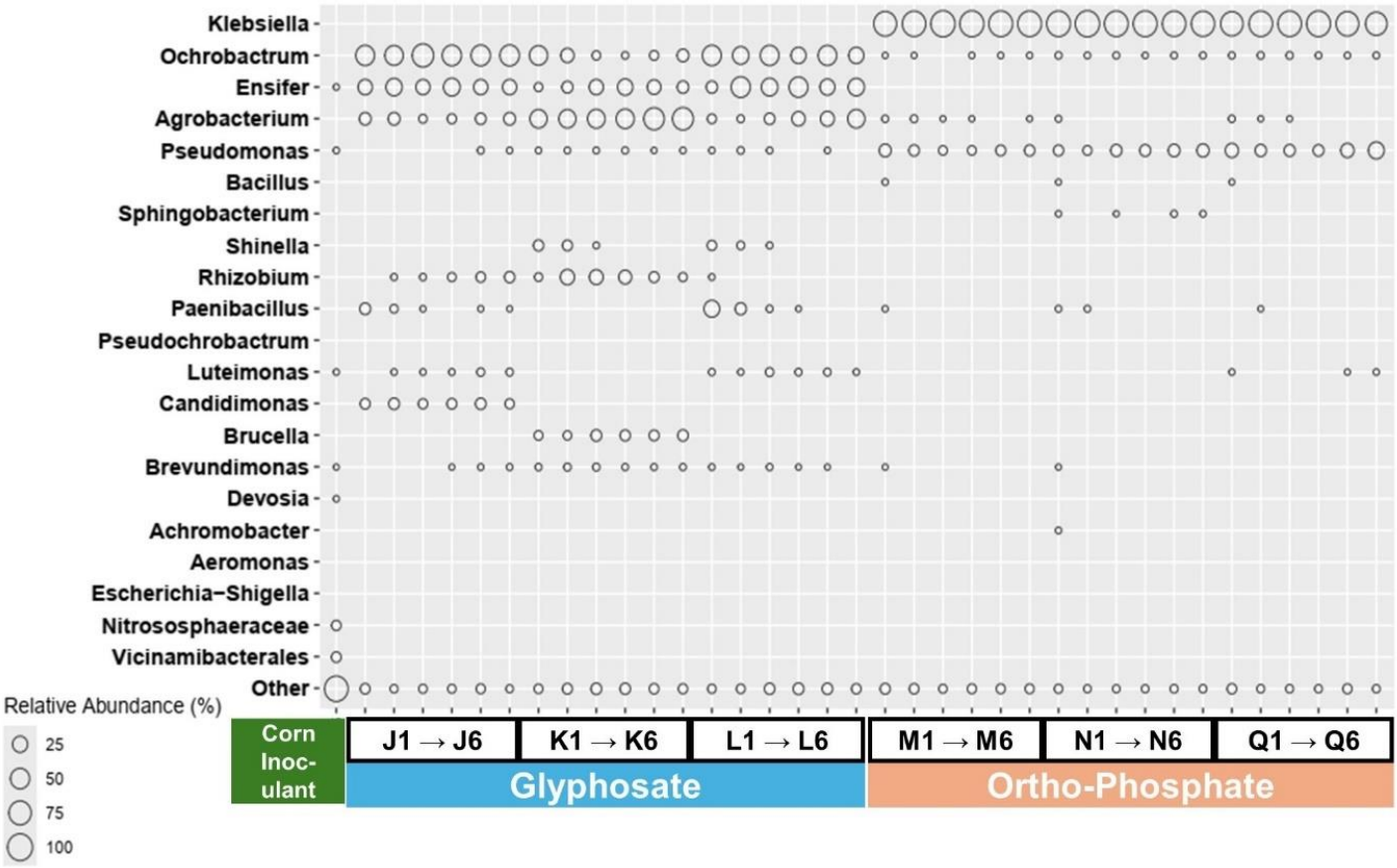


Figure 3.4 Relative Genus Abundance of Corn Subcultures: Binning reads by genus again shows strong enrichment by glyphosate for *Ochrobactrum*, *Agrobacterium*, and *Ensifer*, and enrichment by ortho-phosphate for *Klebsiella* and *Pseudomonas*. This is similar to the enrichments seen when soil from a soy field was used as the initial inoculant.

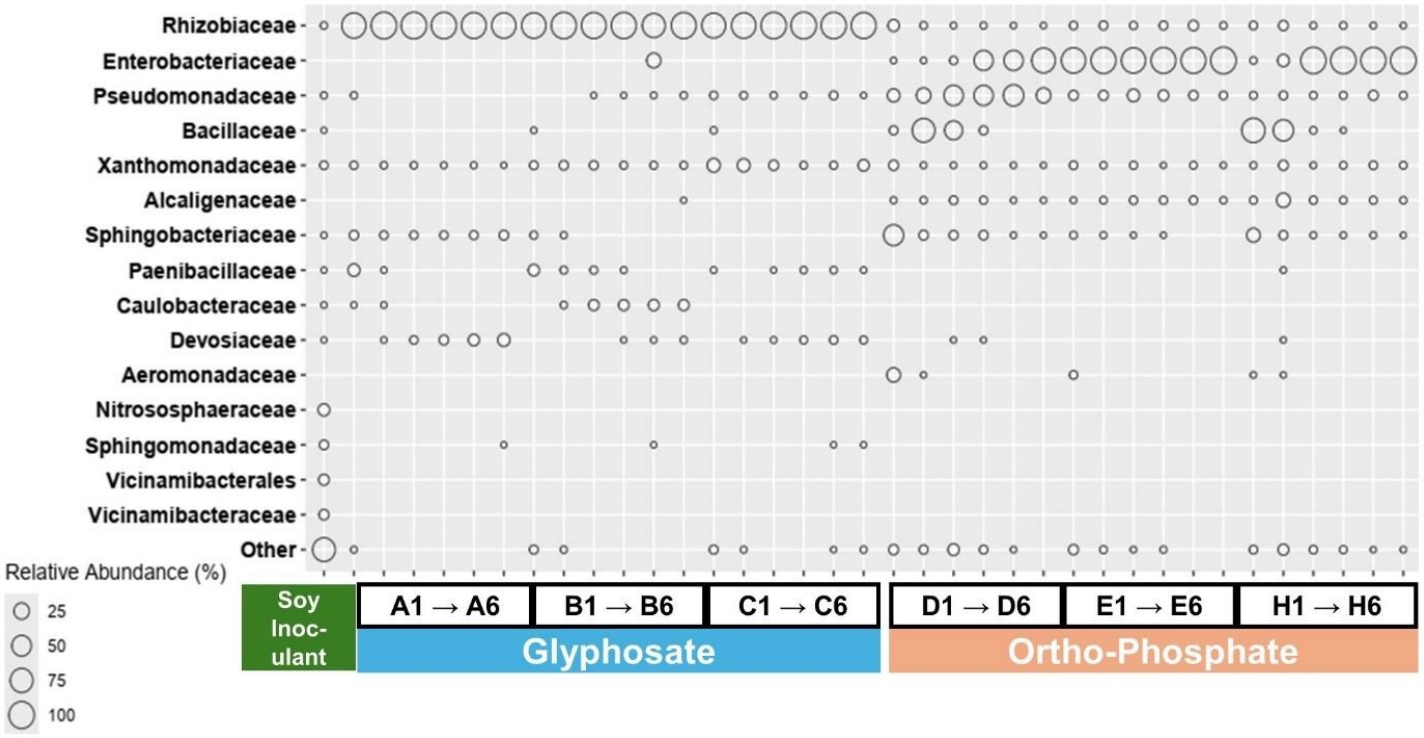


Figure 3.5 Relative Family Abundance of Soy Subcultures: At the family level, Rhizobiaceae dominates the glyphosate subcultures, while Enterobacteraceae and Pseudomonadaceae dominates the ortho-phosphate subcultures.

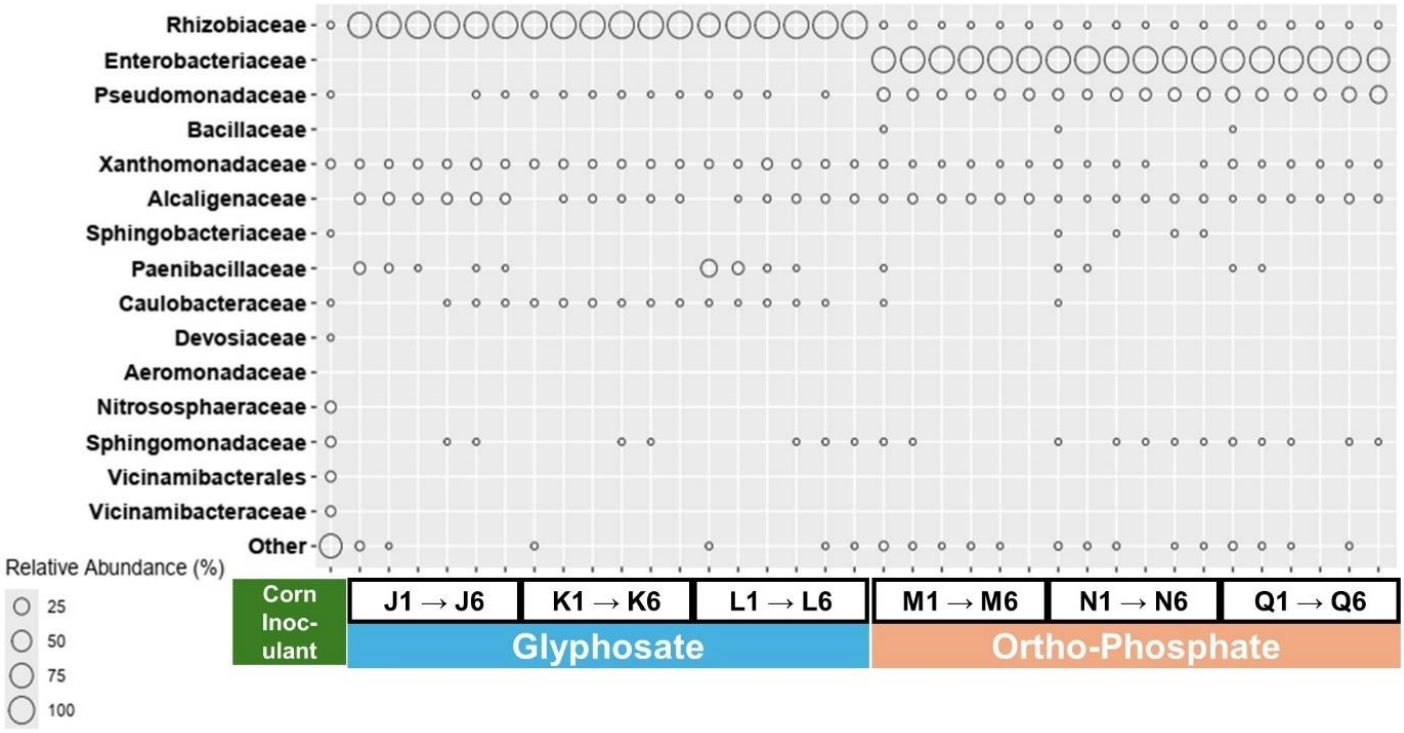


Figure 3.6 Relative Family Abundance of Corn Subcultures: At the family level, Rhizobiaceae dominates the glyphosate subcultures, while Enterobacteraceae and Pseudomonadaceae dominates the ortho-phosphate subcultures. This is a similar pattern to that seen when inoculated with soy field soil.

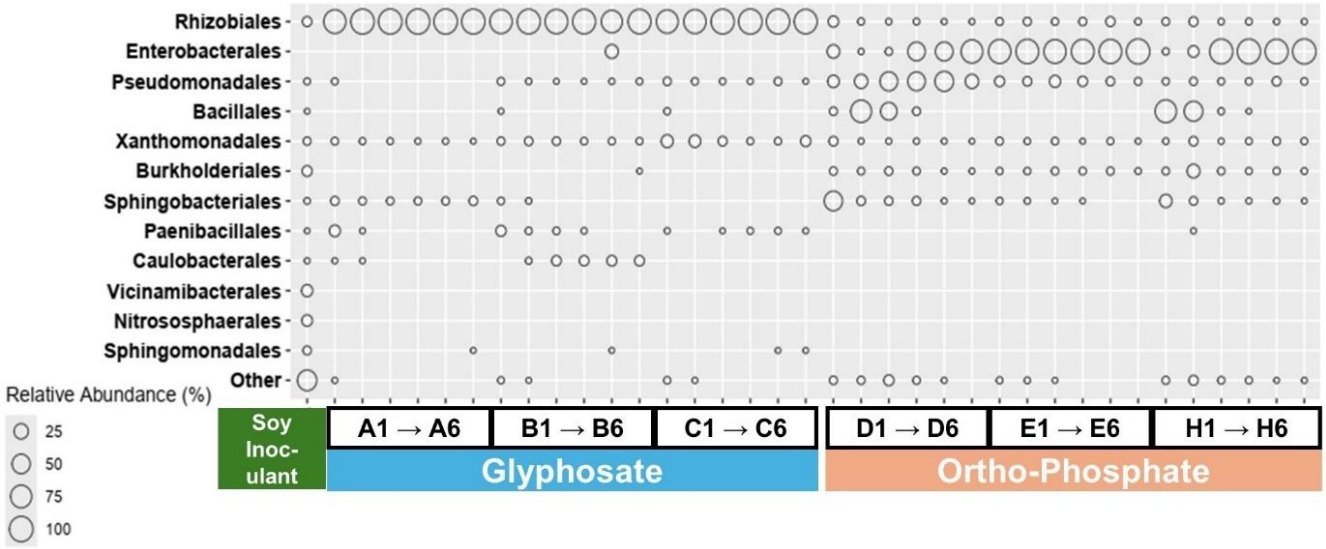


Figure 3.7 Relative Order Abundance of Soy Subcultures: Similar to the family level, Rhizobiales dominate the glyphosate subcultures, while Enterobacterales and Pseudomonadales dominate the ortho-phosphate subcultures.

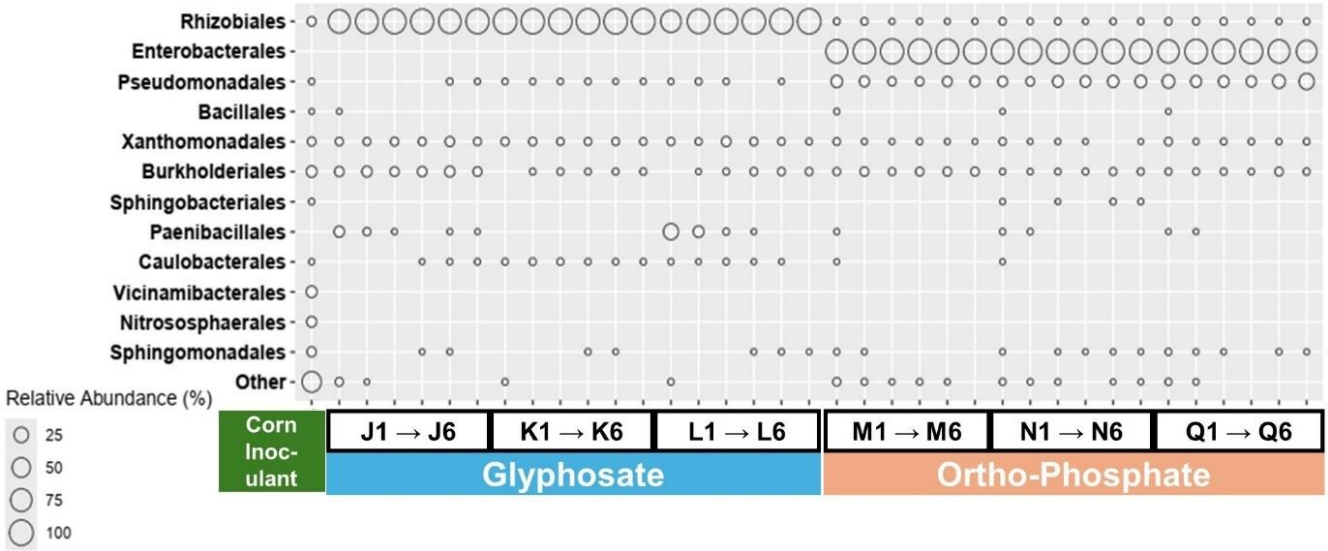


Figure 3.8 Relative Order Abundance of Corn Subcultures: Similar to the family level, Rhizobiales dominate the glyphosate subcultures, while Enterobacterales and Pseudomonadales dominate the ortho-phosphate subcultures.

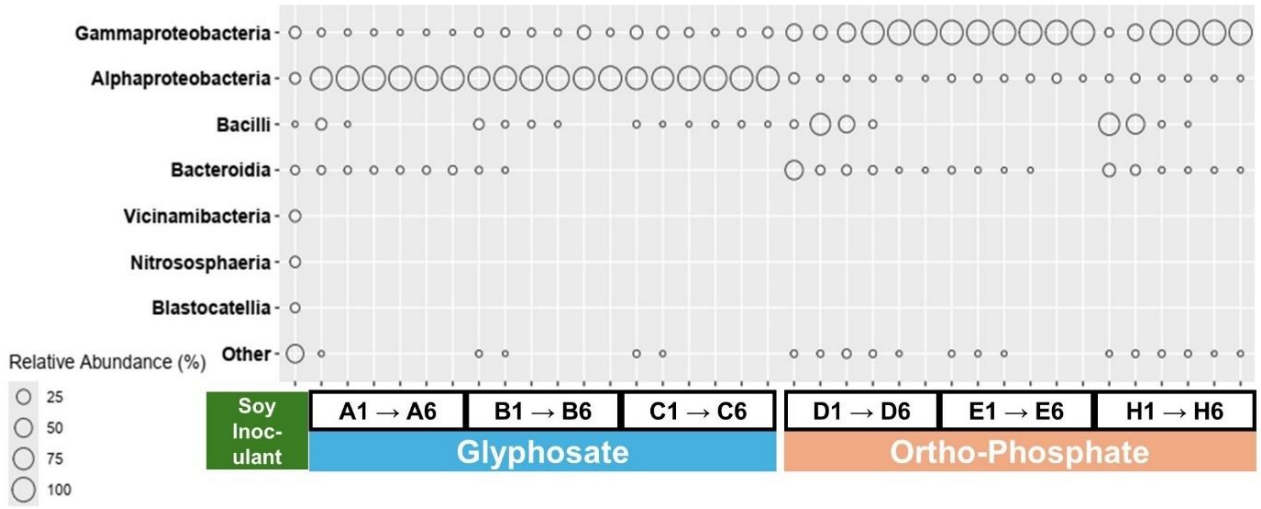


Figure 3.9 Relative Class Abundance of Soy Subcultures: Differences in species enrichment by either glyphosate or ortho-phosphate continue to the class level, with Alphaproteobacteria enriched by glyphosate, and Gammaproteobacteria enriched by ortho-phosphate.

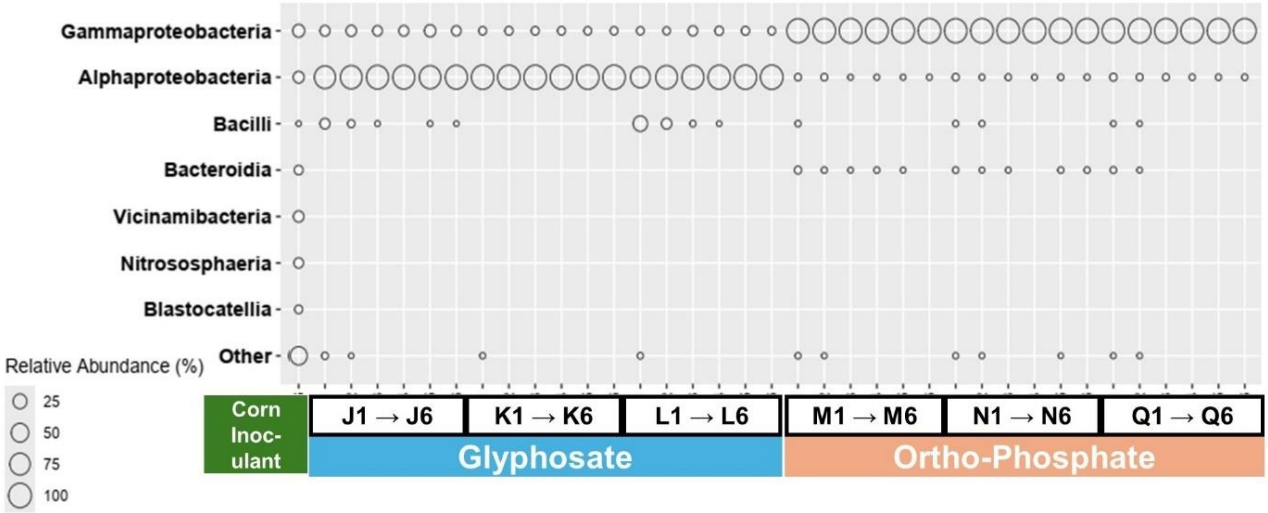


Figure 3.10 Relative Class Abundance of Corn Subcultures: Differences in species enrichment by either glyphosate or ortho-phosphate continue to the class level, with Alphaproteobacteria enriched by glyphosate, and Gammaproteobacteria enriched by ortho-phosphate.

When examining alpha diversity (Figure 3.11), little difference in either the observed species richness or the Shannon's diversity was seen between inoculant sources. Some differences can be seen by phosphorus source, with glyphosate allowing for slightly higher Shannon's diversity, and therefor higher species evenness, compared to the more easily accessible ortho-phosphate. All enrichments showed a decrease in observed species and Shannon's diversity as the enrichments progressed, especially when compared to the original soil used as the inoculant.

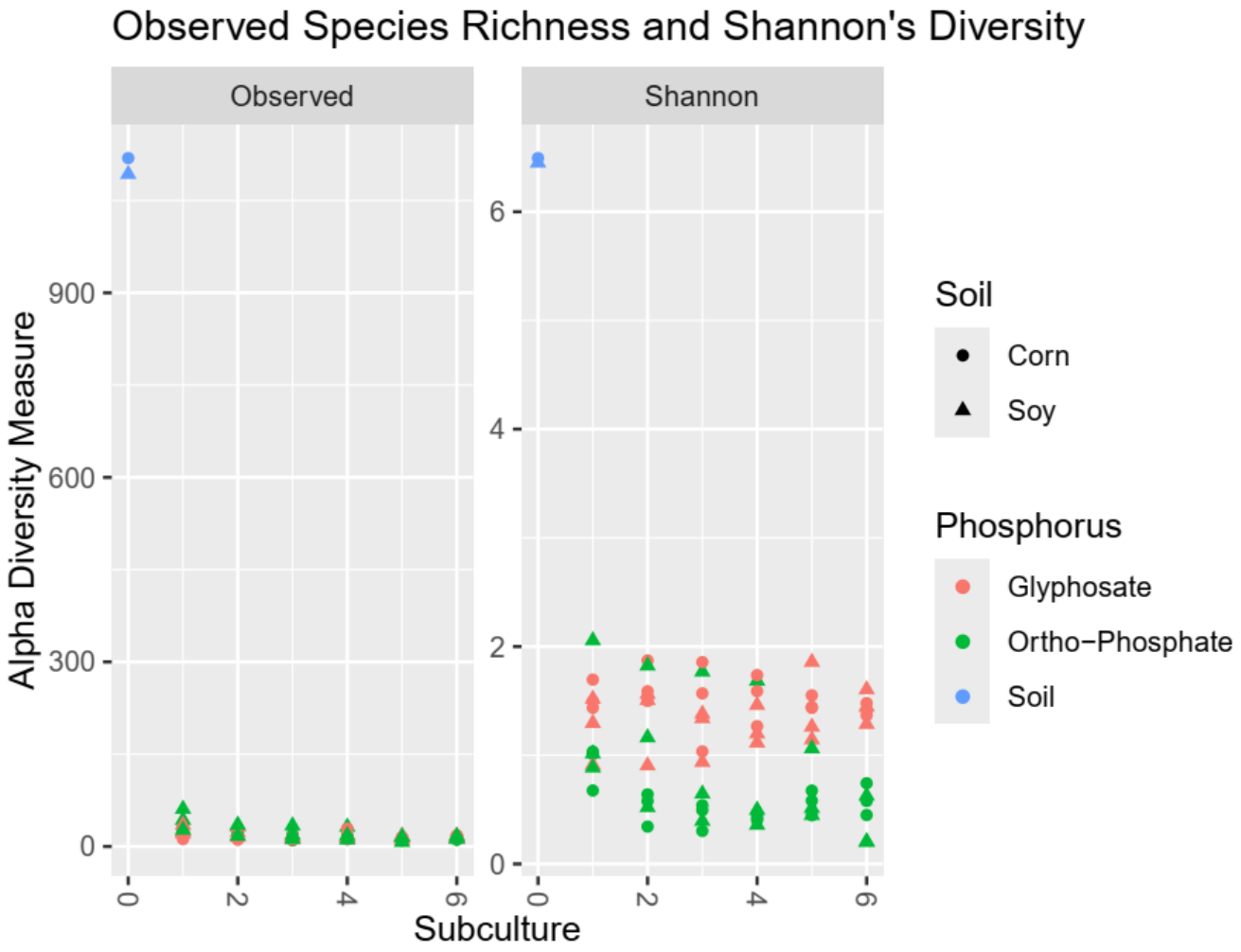


Figure 3.11 Alpha Diversity – Observed Species Richness and Shannon's Diversity: Subculture number shows the order of subcultures with subculture 1 being used to inoculate subculture 2, and so forth. Soil used as initial inoculant is shown as shape, and phosphorus source is shown as colour. Each soil source and phosphorus combination (glyphosate-soy, glyphosate-corn, ortho-phosphate-soy, ortho-phosphate-corn) were used to create 3 subcultures, for a total of 12 total subcultures that were transferred 6 times each. All subculture are shown here. Glyphosate and ortho-phosphate both allow for similar alpha diversity within samples, however glyphosate allows for a larger of Shannon's diversity as compared to ortho-phosphate. Both phosphorus sources show a significant drop in both observed species Shannon's diversity compared to the original soil (shown as subculture 0).

Ordination plots were used to examine beta diversity, the differences between samples. Both Bray-Curtis and Weighted UniFrac were used and both measures showed strong differences by phosphorus source and few differences by which soil was used as the inoculant. For both Bray-Curtis and weighted UniFrac, samples are separated on the first axis by phosphorus type. When using Bray-Curtis Axis 2 however shows significant differences between the glyphosate samples and little difference between the ortho-phosphate samples. In contrast, UniFrac shows few differences between the glyphosate samples and instead shows differences between the ortho-phosphate samples on the second axis. Additionally, the UniFrac differences between the ortho-phosphate samples on the second axis are primarily differences between the enrichments inoculated with soil from a soy field. The corn samples show much tighter grouping. Both amendments diverge from the original soil, however ortho-phosphate has some cultures that hold onto some of their similarity with the original soil.

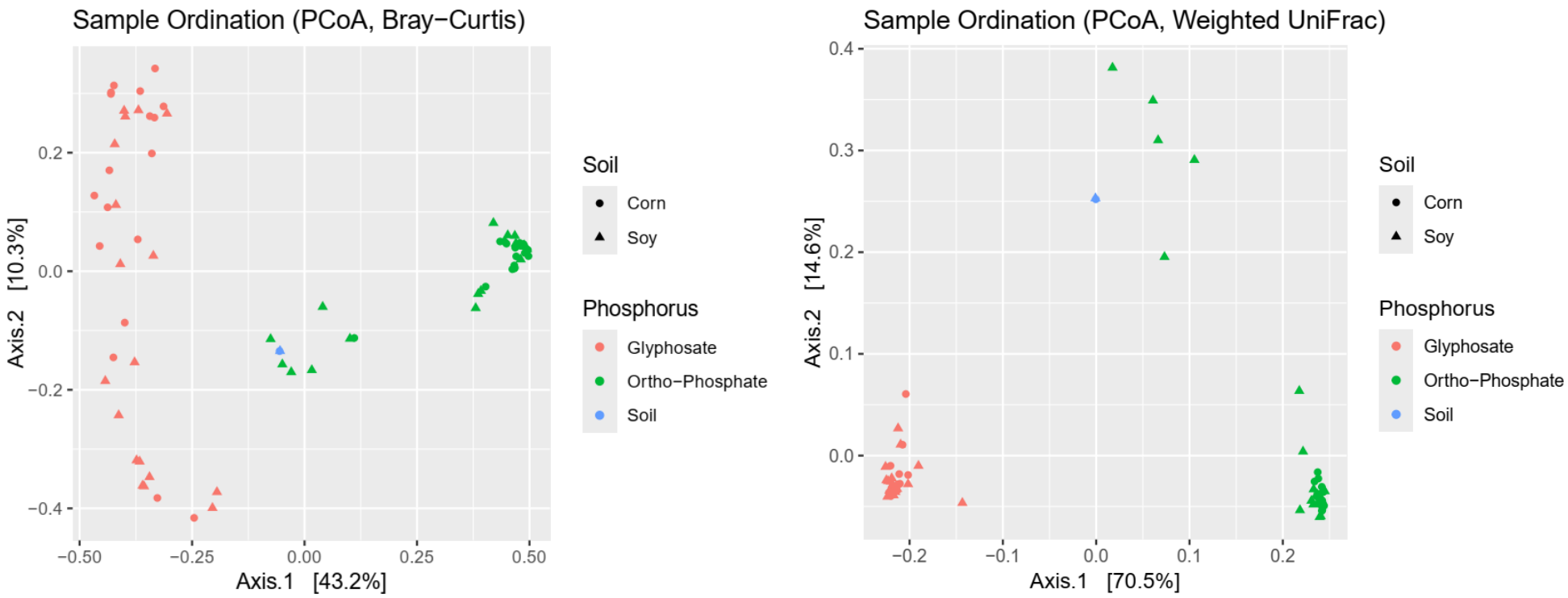


Figure 3.12 Principal Coordinate Analysis: All enrichments are shown using both phosphorus amendments and both soils as initial inoculants. All 6 subcultures within the series of each of the three replicates of each soil-phosphorus combination are shown, along with the initial soil which did not undergo growth in the lab or amendment. Both Bray-Curtis and weighted UniFrac were used to calculate sample ordination. Using both methods, samples group by phosphorus supply, and groups are primarily independent of the soil used to inoculate them. When using Bray-Curtis, the glyphosate samples separate over the second axis while ortho-phosphate remains grouped. Weighted UniFrac instead shows closely grouped glyphosate samples and divergence between the ortho-phosphate samples.

3.3.2 Testing enrichment culture growth

Growth for all 6 subcultures grown on glyphosate as the sole source of phosphorus was re-tested**,** and the first subculture was compared to the 6th (Figure 3.13). All cultures showed growth on glyphosate compared to the uninoculated control, but in general, the sixth subcultures (circular markers) grew significantly better than the first subcultures (triangular markers). This is especially true for the enrichments started with soil from a corn field, in which all three enrichment series showed measurable improvement in both growth (as measured by optical density) and degradation of glyphosate.

A commercial ELISA kit was used to quantify glyphosate left after 96 hours of growth, and this told a similar story to the growth curves. For the enrichments started with corn, there is clearly good degradation of glyphosate by the sixth subculture, but poor degradation by the first subculture. In the case of soy, there is no clear improvement in glyphosate degradation between the first and 6th subcultures. Glyphosate was calculated using a line of best fit from 0.025 g/L to 0 g/L glyphosate because cultures had an initial glyphosate concentration of 0.025 g/L, calculated glyphosate concentrations above this range are an extrapolation.

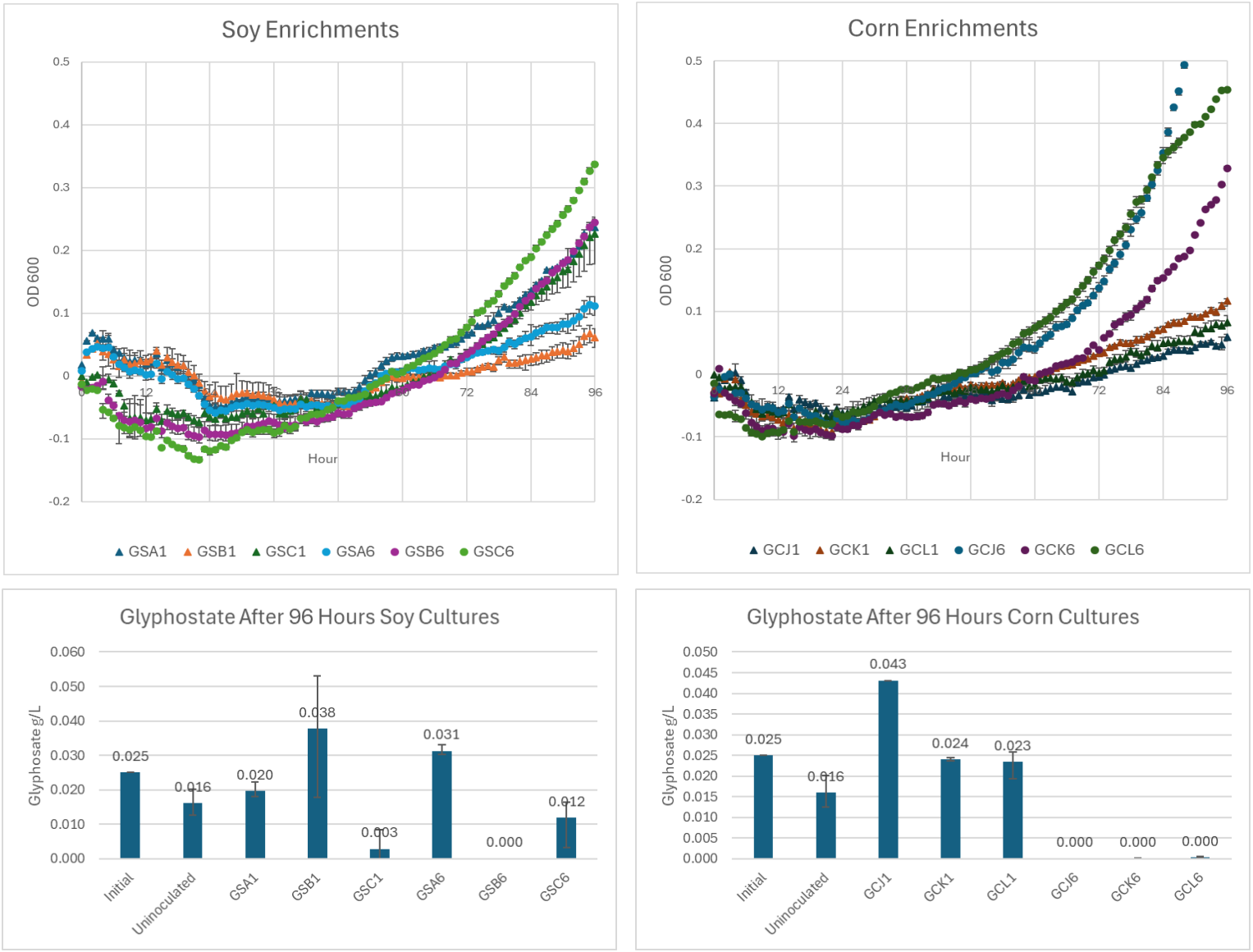


Figure 3.13 Enrichment Growth Tests: Growth on glyphosate and final glyphosate concentration. The first and sixth subcultures were thawed and used to inoculate media with 0.025 g/L glyphosate as sole source of phosphorus. Optical density readings were taken every hour using a BioscreenC growth curve machine. The final concentration of glyphosate was measured after 96 hours using a commercial ELISA assay. In most cases, the sixth subculture grew better than the first, especially the cultures inoculated with soil from a corn field. For the corn cultures, this matches the glyphosate results, showing a significant drop in glyphosate concentration only in the sixth subculture but not the first. The soy cultures did not show this pattern when glyphosate was measured directly. Error bars show the highest and lowest optical density or calculated glyphosate concentration.

3.4 Discussion

3.4.1 Glyphosate and ortho-phosphate enrichment

Enrichments were made using a defined media and either glyphosate or ortho-phosphate (NaH_2PO_4) as the sole source of phosphorus, and with two different inoculant sources (soil either from a corn field or from a soy field). Communities showed strong and reproducible differences by phosphorus source but fewer differences by initial inoculant.

Glyphosate was found to enrich primarily for members of the Rhizobiaceae family of the Alphaproteobacteria class, while ortho-phosphate enriched primarily for a *Klebsiella*, and *Pseudomonas* species, both part of the Gammaproteobacteria class.

Glyphosate degradation by Rhizobiaceae, and enrichment of Rhizobiaceae has been previously reported (Firdous et al., 2018; Massot et al., 2021; Zhumakayev et al., 2021). Unlike this study, previous studies have not been able to differentiate the selective effects of the media and laboratory growth from the selective effects of glyphosate as a source of phosphorus. Though Rhizobiaceae was found in the ortho-phosphate enrichments, it did not dominate like it did in the glyphosate enrichments.

Among the species enriched for by glyphosate are *Ochrobactrum rhizosphaerae* and *Agrobacterium tumefaciens,* and *Ensifer adherens.*

The *Ochrobactrum* genus is known to contain glyphosate degrading species (Firdous et al., 2018). It has been shown that *Ochrobactrum intermedium* Sp20 and *Ochrobactrum anthropi* contain a class II EPSPS that is resistant to inhibition by glyphosate (Firdous et al.,

2018; Tian et al., 2010). Using glyphosate as the sole source of both carbon and phosphorus, it was shown that complete mineralization of 0.5 g/L glyphosate by *Ochrobactrum intermedium* Sp20 can be accomplished in as little as 105 hours (Firdous et al., 2018). *Ochrobactrum anthropi* is known to contain a functional C-P lyase system and multiple studies have shown its ability to degrade glyphosate and use it as a source of phosphorus (Firdous et al., 2018; Hove-Jensen et al., 2014). Thus, it is not surprising that related *Ochrobactrum* species were enriched when selecting with glyphosate as the sole source of phosphorus. Because of the role of glyphosate in agricultural weed control and crop protection, it is worth noting that *Ochrobactrum anthropi* has been shown to provide plant growth promoting effects ranging from nutrient solubilization, to disease prevention, to nitrogen fixation (Meng et al., 2014). If *Ochrobactrum* enrichment also occurs in soils exposed to glyphosate, it may provide a slight plant growth promotion affect.

Another major constituent of the glyphosate enrichments was *Agrobacterium tumefaciens*. Like *Ochrobactrum*, *Agrobacterium* generally contains a glyphosate resistant class II EPSPS. It was in fact *Agrobacterium tumefaciens* CP4 that provided the resistant EPSPS that was engineered into the majority of glyphosate resistant crops (Green & Owen, 2011). Both the related *Agrobacterium radiobacter* and *Agrobacterium tumefaciens* have been shown to contain the C-P lyase operon and be able to degrade glyphosate for use as a source of phosphorus (Hove-Jensen et al., 2014; Massot et al., 2021). Though *Agrobacterium* are often plant pathogens, not all strains are pathogenic, and not all isolates contain the plasmids responsible for pathogenicity (Flores-Félix et al., 2020; Weisberg et al., 2022).

Ensifer adherens was also enriched in the glyphosate subcultures. It has been shown that the related *Ensifer meliloti* is able to use glyphosate as a source of phosphorus (Hove-Jensen et al., 2014; Parker et al., 1999). Interestingly, a knockout of the C-P lyase in *Ensifer meliloti* was shown to prevent growth on glyphosate as sole phosphorus source (Parker et al., 1999). Previous work has also shown *E. adherens* to be a plant growth promoter and to be glyphosate tolerant (Zhumakayev et al., 2021). Though they did not test glyphosate degradation, *E. adherens* was isolated from soil by selecting for glyphosate tolerance, and was shown to produce increased root growth in tomato seedlings, as well as synthesizing plant growth promoting indol-3-acetic acid, and siderophores (Zhumakayev et al., 2021).

3.4.2 Community composition and alpha diversity

The diversity of both the glyphosate degrading and ortho-phosphate communities was also examined, both within communities and between them. The number of observed species, or richness, of each treatment was roughly similar, dropping quickly from the high diversity of the inoculant and continuing to decrease towards the 6th subculture. A significant drop in diversity is expected with enrichment, and even necessary if researchers aim to study simplified communities able to perform a function of interest (Jiao et al., 2016). The continued drop in observed species is likely due to marginal members of the community dropping out as the enrichment progresses and matures. Slow growers were also likely lost owing to a short transfer time of twice a week. Though slow growers were lost, a short transfer time reduced the ability of liberated phosphorus to transfer through the community.

Long growth periods would have likely resulted in phosphorus being liberated from glyphosate by a glyphosate degrading organism, and then eventually re-used by species incapable of degrading glyphosate.

Though observed species counts the number of species present, Shannon's diversity which takes species evenness into account (Wagner et al., 2018). In contrast to observed species, Shannon's diversity does show a difference between the glyphosate subcultures and those provided with ortho-phosphate. Glyphosate as the sole source of phosphorus allows for more species evenness, while evenness is lower in the ortho-phosphate subcultures. This can also be seen in the graphs of species relative abundance (Figure 3.1 and 3.2), in which the ortho-phosphate communities come to be dominated by a single species of *Klebsiella* with a minor contribution by *Pseudomonas* species, while the glyphosate cultures support *Agrobacterium*, *Ochrobactrum*, and *Ensifer* species at comparatively equal proportions. As stated earlier, *Agrobacterium*, *Ochrobactrum* and *Ensifer* both include species that have been shown to liberate phosphorus from glyphosate (Firdous et al., 2018; Hove-Jensen et al., 2014). Generally phosphorus liberation from glyphosate is performed by the self contained C-P lyase pathway in these organisms and is not a multi-species endeavor (Hove-Jensen et al., 2014). As such, the glyphosate degrading consortium likely contains members that are fully able to degrade glyphosate on their own, but are also able to exist as a portion of a diverse community rather than coming to dominate it. By contrast, when ortho-phosphate is the sole source of phosphorus, a single taxa of *Klebsiella* comes to dominate the community, increasing in proportion as the subculture continues. While both sources of phosphorus allow

similar numbers of species to survive (observed taxa), glyphosate allows more species to thrive within the community relative to their peers, shown by higher Shannon's diversity. This may be due to glyphosate degradation being a relatively slow process, which slows the growth of all species in the glyphosate enrichments and lets more species grow at relatively similar levels. In contrast, it may be that easily accessible orthophosphate encourages the fastest growers (*Klebsiella*) to out grow all others and thereby dominate the culture before other species have the opportunity for significant growth.

Species identification was performed using a SILVA database, which identified many species within the Rhizobiaceae family *Allorhizobium-Neorhizobium-Pararhiozobium* (Quast et al., 2012). This is due to the frequent reorganization of this family leading to difficulty keeping databased updated (Mousavi et al., 2014, 2015). For clarity within this study, Rhizobiaceae species were therefore further identified using BLAST and the actual V4 sequence.

3.4.3 Beta diversity and ordination

Beta diversity was measured using two methods, Bray-Curtis dissimilarity, and weighted UniFrac. While Bray-Curtis looks at the dissimilarity between communities from a pure numbers perspective, weighted UniFrac incorporates both species counts and the phylogenetic distance between species (Lozupone & Knight, 2005; Su, 2021). Both measures gave similar but not identical results.

Both Bray-Curtis and weighted UniFrac showed strong clustering by phosphorus amendment, and in general did not show strong clustering by soil type. Bray-Curtis showed a stronger spread over axis 2 of the glyphosate enrichments, reflective of their varying species proportions (of the same species) and of the variability of the members within those enrichments. The ortho-phosphate cultures by comparison showed strong clustering under Bray-Curtis, reflecting the fact that they were all dominated by a single species of *Klebsiella*. Taking species phylogenetic relationships into account with UniFrac, accounted for even more difference between the two amendments, with strong grouping along the first axis, explaining 75% of the differences. Again, communities clustered by amendment type. Weighted UniFrac also showed stronger clustering within the glyphosate communities than Bray-Curtis did, which can be explained by the communities being dominated by closely related species within the Rhizobiaceae family. The increased distance between the ortho-phosphate enrichments and the glyphosate enrichments when plotted using weighted UniFrac reflects the pattern of glyphosate selecting for Alphaproteobacteria while ortho-phosphate selected for Gammaproteobacteria.

3.4.4 Glyphosate degradation over time, between samples

Glyphosate was quantified using an ELISA quantification kit from Gold Standard Diagnostics Horsham. Work by other labs has shown that ELISA is of comparable accuracy to liquid chromatography when quantifying glyphosate, especially when examining complex biological samples (Vélot et al., 2022). ELISA was chosen rather than liquid chromatography

due to its accessibility to the average microbiology lab, in the hope that this work will provide an easily replicable set of methods to find and test additional glyphosate degrading enrichments and species.

Glyphosate degradation was found to improve as the subcultures matured. In general, subculture 6 grew better than subculture 1, reaching a higher optical density in 96 hours. Improved growth was likely due to a combination of improved glyphosate degradation allowing for more growth, and to improved growth in the media generally allowing for a higher optical density even if the same amount of glyphosate is degraded. In the case of the subcultures started from soil from a corn field, there was also clear improvement in glyphosate degradation between subcultures 1 and 6, with subculture 1 degrading very little of the initial 0.025 g/L glyphosate, and subculture 6 degrading all or nearly all of it. By contrast, the cultures inoculated with soil from a soybean field did not show a strong increased ability to degrade glyphosate. It is unlikely that the soy cultures cannot degrade glyphosate effectively because *Agrobacterium* strains were isolated from them that are able to fully degrade the glyphosate (Chapter 4).

Despite the differences in growth results, most enrichment cultures, especially by the 6^{th} subculture, were shown to degrade glyphosate. Enrichments GSC1, GSB1, GCJ6, GCK6, GCL6 were able to degrade all or nearly all of the 0.025 g/L initial glyphosate in 96 hours. It is likely that with additional optimization both to the communities, the strains, and possibly the media, more glyphosate could be degraded. Members of these communities have been shown to degrade glyphosate as isolated species both in this report (Chapter 4) and in the

literature. In particular *Ochrobactrum intermedium*, related to the *Ochrobactrum rhizosphaerae* found in these enrichments, was able to degrade 0.5 g/L glyphosate in 105 hour in a 2018 study by Firdous et al.

Experimental errors, including unhealthy initial inoculant and inaccuracies in the ELISA kit, are likely to blame for the poor glyphosate degradation by GSA6 and GSC6. Inoculant was thawed from the -70°C freezer and underwent a phosphorus exhaustion step; this likely changed the composition of the enrichments and did not promote a healthy inoculant.

Adding to the growth challenges, growth had to be done using a very low concentration of glyphosate, 0.025 g/L, rather than the 1 g/L used in the enrichment. This is because a previous test using 1 g/L glyphosate (unreported) showed no measurable difference in glyphosate concentration post growth, despite an increase in optical density. Tests using pure cultures of glyphosate degrading *Agrobacterium* (section 4.3.7) showed 0.025 g/L to be approximately the point at which glyphosate (phosphorus) became limiting for growth.

The ELISA itself also contained inaccuracies, likely due to the necessity of diluting the media 10^{-4} from 0.025 g/L to 2.5 ppb (0.0000025 g/L) to get it within range of the kit. Though all glyphosate growth curves started with the same defined phosphorus media with 0.025 g/L glyphosate, and this same media was used to make the standard curve, some of the final glyphosate concentrations were measured at greater than the initial 0.025 g/L. These measurements are therefore erroneous.

3.5 Conclusions

This media is useful for finding additional glyphosate degrading organisms from enrichment cultures, using soil as an initial inoculant. A commercial ELISA kit was found to be effective at quantifying glyphosate in bacterial cultures and serves as an accessible method for microbiology labs to quantify the glyphosate degrading potential of glyphosate degrading organisms.

Glyphosate as sole source of phosphorus was found to reliably select for Rhizobiaceae, while ortho-phosphate was found to select primarily for a single species of *Klebsiella* and multiple species of *Pseudomonas*. Additionally, higher Shannon's diversity was observed in the enrichments grown on glyphosate. The addition of glyphosate adds a potential nutrient source, as well as a selective pressure that changes bacterial communities, but may preferentially select for Rhizobiaceae, a family containing a significant number of plant growth promoting strains. Overall, the addition of an easily accessible phosphorus source rather than a difficult to access phosphorus source was the limiting factor in bacterial diversity within the context of these enrichment cultures. Glyphosate did not have a larger impact on bacterial diversity than ortho-phosphate, despite the fact that glyphosate is known to inhibit some bacterial species.

An ordination plot using weighted UniFrac showed that the communities selected by glyphosate and by ortho-phosphate differed greatly, and analysis of the common species in each enrichment showed themselves showed strong selection at the class level of phylogeny. Glyphosate selected for Alphaproteobacteria, while ortho-phosphate selected primarily for

Gammaproteobacteria. Glyphosate is a new molecule in the context of bacterial evolution, but it appears that evolutionary patterns laid down long ago are echoed in the species able to survive under these enrichments.

From a bioremediation perspective, select glyphosate enrichment cultures were able to fully degrade 0.025 g/L glyphosate. The extent to which this degradation is due to the role of the community verses the individuals remains an open question.

Chapter 4

Glyphosate Degrading *Agrobacterium*

4.1 Introduction

4.1.1 Strain comparison and the *Agrobacterium* genus

Historically, *Agrobacterium* were isolated and studied due to their capacity to cause crown gall disease or hairy root disease in plants, including agronomically important crops (Weisberg et al., 2023). Both of these processes are considered oncogenic, caused by a tumor inducing (pTi) or root inducing (pRi) plasmid, which harbor genes necessary to move the plasmid into plant cells and then cause the eukaryotic plant cells to transcribe and express additional genes provided by the oncogenic plasmid for the benefit of the *Agrobacterium* (Nester, 2015; Weisberg et al., 2023). The excessive growth of the plant is due to the plant synthesizing the plant growth hormones auxin and cytokinin, which produces tumor-like structures (galls in the case of pTi) that provide a protected environment for the infecting *Agrobacterium* (Nester, 2015). Additionally, *Agrobacterium* has the capacity to transfer its oncogenic plasmids (and possibly other plasmids or portions of its genome) between *Agrobacterium* strains, allowing for large variation within the *Agrobacterium* species complex (Nester, 2015; Weisberg et al., 2023).

Over its more than 100 year history of study, the *Agrobacterium* genus has gone through multiple reorganizations, and is currently considered part of a larger Agrobacterium-Allorhizobium-Rhizobium group, which is split into three biovars (Nester, 2015; Weisberg et

al., 2023). Originally, the biovars were split based on phenotypic characteristics such as the ability to harbor plant tumor forming plasmids, however recently the biovars have been classified based on comparisons of the phylogeny of marker genes. This reorganization was necessary because functions were found not to be monophyletic and it was found that they could transfer between distantly related strains (Weisberg et al., 2023). In the case of true *Agrobacterium* (biovar 1 and biovar 1-like) genome arrangement is consistent, with true *Agrobacterium* having a circular chromosome, a linear *chromid*, and possibly containing one or multiple additional plasmids (Weisberg et al., 2022, 2023). The chromid is so named because it is unclear if it is essential and should be classified as a chromosome, or if it is more akin to a megaplasmid. It appears that the chromid evolved from a plasmid, and portions of the traditional chromosome moved into it. Significant rearrangement between and within the chromid and plasmids is also seen, again complicating the story of *Agrobacterium* phylogeny (Weisberg et al., 2023).

4.1.2 Glyphosate degradation and the C-P lyase operon in *Agrobacterium*

Agrobacterium has also been found to be capable of using glyphosate as a source of phosphorus, and the C-P lyase pathway has been implicated (Hove-Jensen et al., 2014; Masotti et al., 2021). While glyphosate degradation by C-P lyase can take one of two routes (direct degradation or pre-processing to AMPA) direct degradation has been found in members of the Rhizobiaceae family (Liu et al., 1991; Stosiek et al., 2020). *Agrobacterium* tend to contain class II EPSPS within their aromatic amino acid pathway, which is resistant

to glyphosate (Green & Owen, 2011). Of relevance to the C-P lyase pathway, class II EPSPS does not benefit from glyphosate being degraded to AMPA, even though this is sometimes the first to pre-process glyphosate for degradation by the C-P lyase pathway (Stosiek et al., 2020). Though pre-processing to AMPA in other species may serve as a resistance mechanism, it may not be necessary in *Agrobacterium* if the target of glyphosate is already glyphosate resistant.

C-P lyase initially was discovered in *E. coli* with the gene arrangement *phnCDEFGHIJKLMNOP* (Hove-Jensen et al., 2014). Different arrangements and different copy numbers predominate in other species (Hove-Jensen et al., 2014). Despite different operon structure and arrangement, functions are often grouped. *Agrobacterium radiobacter* and *A. tumefaciens* have the arrangement *phnFGHIJKLO- phnCDE$_1$E$_2$ -duf1045-phnMN* (Hove-Jensen et al., 2014; Masotti et al., 2021). With the first section, *phnFGHIJKLO,* containing the repressor *phnF*, followed by C-P lyase complex genes *phnGHIJKL*, and an accessory gene *phnO*. *A. radiobacter* then has a space in its C-P lyase operon, followed by *phnCDE$_1$E$_2$ -duf1045-phnMN* (Hove-Jensen et al., 2014). Transporter genes *phnCDEE* follow the Rhizobiaceae pattern rather than the single copy of *phnE* in *E. coli* (*phnCDE)*. An additional C-P lyase complex gene is encoded by *phnM*, and an accessory protein is encoded by *phnN* (Hove-Jensen et al., 2014). The gene *duf1045* is also found in *Ensifer meliloti* and *Ochrobactrum anthropi* (also members of the Rhizobiaceae family) and may substitute for *phnP* as *phnP* is absent in *Agrobacterium* (Hove-Jensen et al., 2014; Masotti et al., 2021). Despite a decade of progress since the Hove-Jensen review in 2014, the function of Duf1045

is still unknown. A glyphosate degrading *A. tumefaciens* has also been shown to share this gene structure (Masotti et al., 2021).

4.1.3 Further research into *Agrobacterium* and glyphosate degradation

Because of its possible implications in plant disease, its extraordinary genome flexibility, and its ability to degrade glyphosate, *Agrobacterium* is clearly worthy of additional study. To that end, the genomes of 4 strains of glyphosate degrading *Agrobacterium* (consisting of 3 distinct species) were fully sequenced and analyzed. One strain, *Agrobacterium pusense* RIM-Id RfA was isolated from the hydroponic system of a research greenhouse (in an unrelated study) and was later found to be capable of growing on glyphosate as a sole source of phosphorus. This strain was selected for further tests presented here, including a gene knockout and replacement experiment to elucidate the role of C-P lyase. *Agrobacterium tumefaciens* strains GSA6-1 and GSC6-1 were both isolated from enrichment cultures (Chapter 2) that were selected for their ability to grow on glyphosate as a sole source of phosphorus. Additional isolates were isolated from these enrichment cultures but sequencing revealed all strains to be identical to one of two closely related strains. GSA6-1 and GSC6-1 were selected as representatives of these two strains. Additionally, a rifampicin resistant mutant of the well-studied *Agrobacterium fabrum* C58 RfB was sequenced and compared. While initial testing indicated that *A. fabrum* C58 was a poor glyphosate degrader, further testing revealed that it was a slow grower that would grow on glyphosate as the sole source of phosphorus if given enough time. Though *A. fabrum* C58 is well studied, and was in fact

one of the first *Agrobacterium* to be sequenced, re-sequencing was justified on the basis of ensuring accurate genome comparisons between the exact isolates tested in this study (Wood et al., 2001).

In order to compare the genomes of these strains, average nucleotide identity (FastANI) was used to determine which strains were unique, and multiple genome alignment was used (progressiveMauve) to look at genome rearrangements between strains (Darling et al., 2010; Jain et al., 2018). Genomes were annotated using RAST and the C-P lyase operons were confirmed and compared manually (Brettin et al., 2015).

Though C-P lyase has been implicated as the glyphosate degrading pathway in *Agrobacterium*, it appears that as of yet knockout experiments have not been performed (Hove-Jensen et al., 2014; Masotti et al., 2021). There is a need for additional testing to prove the role and necessity of C-P lyase in glyphosate degradation by *Agrobacterium*. To that end, homologous recombination was used to replace the C-P lyase genes *phnGHIJ* of *A. pusense* RIM-Id RfA with the spectinomycin/streptomycin resistance gene from pHP45Ω-SmR/SpR. This C-P lyase deficient *A. pusense* was confirmed and tested for growth on glyphosate as sole source of phosphorus.

4.1.4 Genomic library creation

While the ability to degrade phosphonates can provide bacteria with much needed phosphorus when none is otherwise available, the ability to break down phosphonates

generally and glyphosate specifically is not ubiquitous. Future work should be devoted to elucidating what is deficient in strains that are unable to degrade glyphosate as a sole source of phosphorus. A solution to finding these deficiencies could be through genetic complementation experiments, in which the missing genes or pathways are moved from glyphosate degrading strains into related non-degrading strains. To that end, genomic libraries of both *A. tumefaciens* GSA6-1 and *A. pusense* RIM-Id RfA were created.

In part as a proof of concept, and in part to prove C-P lyase is responsible for glyphosate degradation in *Agrobacterium*, the *A. pusense* RIM-Id RfA library was moved into a C-P lyase deficient *A. pusense* RIM-Id RfA Δ*phnGHIJ*. Using this library, a clone was selected that restored glyphosate degradation in the C-P lyase knockout. The genomic libraries were made using the modified method from Cheng et al 2014 in which clean genomic DNA was isolated from pure cultures, this was ligated to the pJC8 backbone, providing an origin of transfer, *cos* sites, and tetracycline resistance. Phage heads were used to preferentially package pJC8 with inserts approximately 40 kbp long. The phage heads were then transduced into *E. coli* HB101. Because the backbone contains an origin of transfer, movement between Pseudomonadota (formerly Proteobacteria) by conjugation is simple, including movement into the knockout *A. pusense* RIM-Id RfA Δ*phnGHIJ*. The library should be able to move into and express in a wide variety of Pseudomonadota, especially those in the Rhizobiaceae family (Cheng et al., 2014).

4.1.5 Overview

Here, the genomes of 4 strains of *Agrobacterium* and their ability to degrade glyphosate are examined. To study this, strains were fully sequenced, assembled, and annotated. Growth on glyphosate was tested and compared, along with the C-P lyase operon of each of the strains. Further examination of the C-P lyase pathway revealed that knocking out C-P lyse stops glyphosate degradation in *A. pusense*, while replacing it restores function, showing that C-P lyase is in fact the pathway used to degrade glyphosate.

4.2 Materials and Methods

4.2.1 Materials, antibiotics, and plasmids

The ABRAXIS glyphosate ELISA and derivatization kit used to quantify glyphosate was purchased from Gold Standard Diagnostics Horsham, of Horsham, PA, USA. Chemicals used to make the media were purchased from Bio Basic, with the exception of; magnesium sulfate, purchased from BioShop Canada; and glyphosate, purchased from Toronto Research Chemicals.

Table 4-1 Antibiotics Used in This Chapter

Antibiotic	Concentration µg/mL	Abbreviation
Neomycin	100	Nm100
Rifampicin	50	Rif50
Spectinomycin	100	Spec100
Tetracycline	10, 20	Tet10, Tet20
Chloramphenicol	5	Cm5
Nalidixic acid	30	Nal30

Above are the selective antibiotics used in this chapter, along with their final concentration and abbreviation. Generally, antibiotics are diluted from 1000X their final concentration into either liquid media or melted agar.

Table 4-2 Plasmids Used in This Chapter

Plasmid	Resistance	Function	Source
pRK600	Chloramphenicol	Helper plasmid for tri-parental mating	(Finan et al., 1986)
pK19mobsacB	Kanamycin/Neomycin	Counter-selectable plasmid for homologous recombination	(Schäfer et al., 1994)
pJC8	Tetracycline (backbone) Gentamicin (stuffer)	Host cosmid for metagenomic library creation	(Cheng et al., 2014)

Plasmids used, along with their resistance and function are given above. It is important to note that pJC8 used to create the genomic library initially contains a stuffer gene (gentamicin resistance) that is removed before genomic DNA is ligated into it.

4.2.2 Initial *Agrobacterium* isolation and screening for growth on glyphosate

Using the defined phosphorus media previously described (Section 2.2.2), a portion of the collection of strains within the lab were initially screened for glyphosate degradation. Strains were tested for their growth in liquid culture using base media without phosphorus (negative control), with glyphosate as the sole source of phosphorus (to test for glyphosate degradation), with glyphosate and ortho-phosphate (to test for resistance to glyphosate), and with ortho-phosphate added alone (as a positive control to test our media).

Additionally, the glyphosate enrichment cultures started with soil from a soy field (GSA, GSB, GSC) from Chapter 3 were used to isolate additional strains. Pure cultures were isolated by plating on defined phosphorus media with glyphosate as the sole source of

phosphorus and Noble agar as the gelling agent. Strains were streak purified and then confirmed for glyphosate degradation using the same procedure as above.

4.2.3 Genome sequencing, assembly, and annotation

Genomes were sequenced using a hybrid approach. With the exception of *Agrobacterium* RIM-Id RfA, long reads were generated using an Oxford Nanopore MinIon and short reads were generated on an Illumina NextSeq. For *Agrobacterium* RIM-Id RfA, long reads were generated using PacBio through the McGill University and Genome Quebec Innovation Centre. Genomes were assembled using Unicycler through the Bacterial and Viral Bioinformatics Resource Center (BV-BRC) (Olson et al., 2023; Wick et al., 2017). Unicyler overcomes the shortfalls associated with the errors inherent to Nanopore long read sequences by first assembling a genome using the long reads, and then correcting it using the highly accurate Illumina short reads (Wick et al., 2017). Annotation was also performed through BV-BRC using the RAST toolkit (Brettin et al., 2015). Assembly completeness was checked within Galaxy using CheckM lineage_wf (Parks et al., 2015). Strains were identified at the species level using GTDB-Tk through Galaxy Europe (Chaumeil et al., 2020).

4.2.4 Strain comparison

For the fully assembled isolates, average nucleotide identity was calculated using FastANI within Galaxy Europe (Jain et al., 2018; The Galaxy Community et al., 2024). Additionally,

fully assembled and unique strains were aligned within BV-BRC using progressiveMauve (Darling et al., 2010).

4.2.5 Knockout procedure, PCR confirmation

To test the necessity of the C-P lyase system for glyphosate degradation in *Agrobacterium* RIM-Id, RfA a knockout was made using homologous recombination (Figure 4.1). The *phnGHIJ* portion of the operon was replaced with the spectinomycin/streptomycin resistance gene (Sp^R/Sm^R) from pHP45Ω-SmR/SpR, using a double crossover. Homologous recombination using the counter selectable plasmid pK19mobsacB was performed. By placing the Sp^R/Sm^R gene between the 300 bp sequence upstream of *phnG* and the 300 bp immediately downstream of *phnJ*, a sequence with homologous regions upstream and downstream to the *phnGHIJ* gene set of *Agrobacterium* RIM-Id was created. This was synthesized by BioBasic and placed in the pK19mobsacB backbone. The pK19mobsacB plasmid contains a kanamycin/neomycin resistance gene along with a sucrose toxicity gene, sacB. pK19mobsacB has a narrow host range oriV origin of replication and is unable to replicate within *Agrobacterium* (Schäfer et al., 1994). Therefore, replication must take place by insertion into the genome. A rifampicin resistant mutant of RIM-Id was selected, and the plasmid was transferred to it using triparental mating and the pRK600 helper plasmid (Finan et al., 1986). The first insertion was selected for by plating transconjugants on tryptic soy agar with Rif-50 and Nm-100, selecting for *Agrobacterium* RIM-Id RfA (Rf^R) and the plasmid pK19mobsacB-RT5 (Km^R/Nm^R) existing as a genome insertion. This selected for a

single crossover. This was restreaked twice to purify, and then streaked on TSA-5% sucrose with Spec100. This selected for double crossovers that knocked out the region between the homologous region containing the genes *phnGHIJ,* as well as removing the plasmid backbone that was previously inserted into the genome.

Knockouts were confirmed using PCR. A pair or primers were located on either side of the deletion (and outside of the homologous regions used to make the deletion) and a second pair using a forward primer from within the deletion and the reverse primer from the first set, primer arrangement is shown in Figure 4.1. The first set produced a 1789 bp amplification if the deletion was successful, and the second set produced a 1074 bp amplification if the deletion was not.

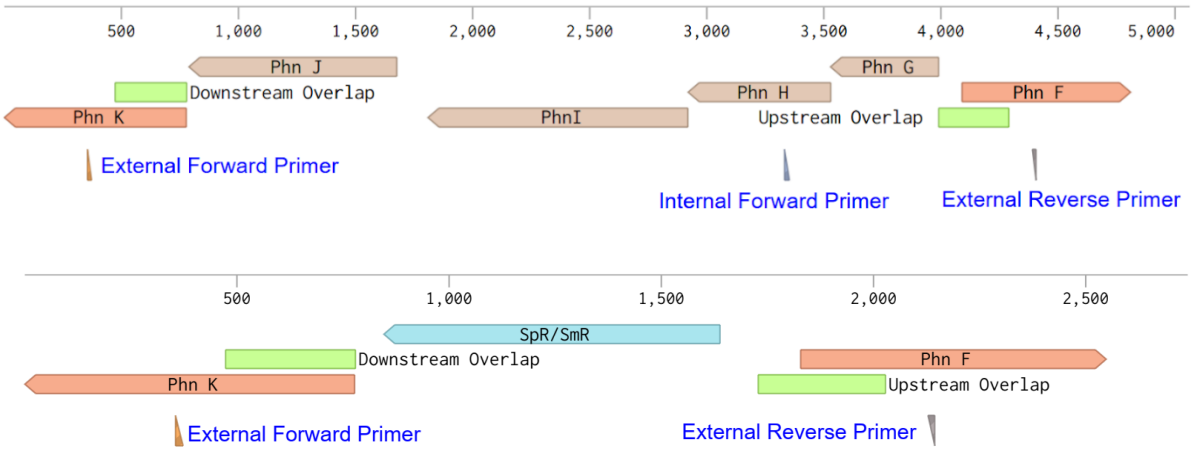


Figure 4.1 *phnGHIJ* Knockout Arrangement: Homologous recombination was used to replace the C-P lyase genes *phnGHIJ* with Sp^R/Sm^R. This was performed using sucrose counter selection and the pK19mobsacB plasmid. Knockouts were confirmed by PCR, using the internal and external primers shown.

4.2.6 Genomic library

Genomic cosmid libraries were made from the glyphosate degrading *Agrobacterium* strains RIM-Id RfA and GSA6-1. As a proof of concept, the RIM-Id RfA library was used to complement the RIM-Id RfA Δ*phnGHIJ* knockout strain. Clones which restored glyphosate degradation restoring clones were selected.

Libraries were made using a modified method from Cheng et al., 2014. When constructing cosmid libraries, it is important to balance the need for clean DNA sample at each step with the inherent loss of product associated with that cleaning. In brief, a stock of pJC8 backbone cosmid was grown and isolated. This was digested by Eco72I and dephosphorylated by alkaline phosphatase (FastAP, Thermo Scientific). Digestion was

confirmed by gel electrophoresis, and dephosphorylation was confirmed by undergoing a test ligation with T4 ligase and confirming low re-ligation rates. A cut but not dephosphorylated control was used as a positive control for ligation. Vector DNA was not gel extracted. After confirmation, cut and dephosphorylated vector was cleaned by isopropanol precipitation (1.25 volumes isopropanol, 0.065 volumes 5 M NaCl, spin 30 minutes at 20,000 rcf, rinse with 70% isopropanol, spin 15 minutes at 20,000 rcf, dispose of supernatant and dry DNA pellet for 15 minutes at room temperature in a flow hood). Vector DNA was redissolved in 1/10th the original volume in 10 mM Tris pH 8.0.

Genomic DNA was isolated from cultures of *A. pusense* RIM-Id RfA and *A. tumefaciens* GSA6-1 using a SOX soil DNA isolation kit (Metagenom Bio Life Science, Waterloo Ontario). DNA was isolated as recommended by the kit with the following exceptions. 5 mL of liquid cultures were pelleted at 10,000 rcf for 5 minutes and supernatant was decanted, the pellet was resuspended in SOX 1. Additionally, bead beating was only performed for 2 seconds. This was done to maximize the length of the genomic DNA fragments, as 40 kbp fragments were needed for later phage head packaging. The isolated genomic DNA was end repaired according to manufacturer's instructions using the Thermo Scientific Fast DNA End Repair Kit. The end repaired genomic DNA was cleaned with isopropanol precipitation as above and dissolved in 20 µL of 10 mM Tris pH 8.0.

Cut, dephosphorylated, and cleaned vector DNA (pJC8) was ligated to end-repaired and cleaned insert DNA (genomic) using approximately a 10:1 molar ratio of vector:insert. This worked out to 4 µg of pJC8 (12kbp) and 1.5 µg of genomic DNA (estimating 40 kbp).

40 kbp was used because this is the target insert size. Ligation was done using T4 ligase from ThermoFisher Rapid DNA Ligation Kit and a total volume of 45 µL, for 48 hours at 16 ℃. The ligation was precipitated with 1.25 volumes of isopropanol and 0.065 volumes of 5 M sterile NaCl, spun for 15 minutes at 20,000 rcf. The samples were decanted and the DNA pellet rinsed with 70% ethanol, which was again spun at 20,000 rcf for 15 minutes. The ethanol was decanted and the pellet was dried for 15 minutes at room temperature in a flow hood with the tube pointing down. Cleaned DNA was redissolved in 10 µL 10mM Tris pH 7.8 at 4 ℃ overnight. 4 µL of ligated and cleaned DNA was added to 1 Gigapack III XL phage head packaging extract (Agilent Technologies, Santa Clara, California) and incubated at room temperature following the manufacturers directions. Gigapack III XL phage head packaging preferentially packages inserts with a total size of 47-51 kbp. This resulted in 500 µL of packaged phage heads. The entire 500 µL was added to 5 mL fresh *E. coli* HB101 grown to log phase (OD600 of 0.93) in lysogeny broth (LB) supplemented with 10 mM $MgSO_4$ and 0.2% maltose. The phage-*E. coli* mixture was allowed to incubate at 37°C for 60 minutes with turning to mix every 15 minutes. The transduced mixture was spun down at 4000 rcf for 10 minutes, and the pellet gently resuspended in 10.5 mL of LB. 1000 µL portions of this were plated on a total of 10 LB-Tetracycline (10 µg/mL) plates. A dilution series to 10^{-9} was also created and 10 µL spots were plated in triplicate on LB-tet10 to determine library size. The library and spot plates were grown at 37 ℃. The following day the libraries were both found to be significantly over the threshold of 50,000 clones, the entirety of each library was resuspended from the plates and diluted to 50 mL of LB-7% dimethyl sulphoxide (DMSO) and saved at -70°C in 1 mL aliquots. To quantify the diversity

of the library, 12 isolated colonies from each library were taken from the spot plates, and grown in 5 mL of LB-tet20 each. Cosmid DNA was extracted from each of these with a Thermo Scientific GeneJET plasmid miniprep kit. This DNA was digested by EcoRI and HindIII and run on a 1% agarose gel, along with pJC8 as a control. Cosmids with the same digest pattern were assumed to be copies of the same insertion event.

4.2.7 Isolating glyphosate degrading cosmids

The genomic library pJC8-RIM-Id was transferred to the knockout *Agrobacterium* RIM-Id RfA *ΔphnGHIJ* using triparental mating, and selection on TSA-Tet10 Rif50 agar plates. The following procedure was used:

E. coli DH5α containing the helper plasmid pRK600 was used to facilitate the movement of the cosmids from the original *E. coli* HB101 host to the *Agrobacterium* recipient. The pRK600 plasmid contains the *tra* genes necessary for the transfer of pJC8, using its origin of transfer (Finan et al., 1986). Mating was done by growing the recipient and helper in either tryptic soy broth or LB-Cm5 respectively, overnight at 30°C or 37°C (respectively) with oscillation. 2 mL of each culture was pelleted by centrifugation at 10,000 rcf for 5 minutes. A library was thawed on ice and pelleted similarly. 500 µL of LB was used to resuspend and mix all three pellets. The mixture was placed on a tryptic soy agar plate without antibiotics and incubated at 30°C overnight. The following morning it was resuspended and plated on 150 mm wide petri plates of TSA-Rf50-Tet10, and a dilution series was made and spot plated on TSA-Rf50-Tet10. After 4 days the spot plate was counted

to calculate transconjugants and the selection plates were resuspended using sterile 0.85% NaCl, then spun down. The library was resuspended in 7% DMSO-TSB and saved in 1 mL portions at -70°C.

To select for transconjugants that restored the ability of *Agrobacterium* RIM-Id RfA *ΔphnGHIJ* to grow on glyphosate as a sole phosphorus source, a library was thawed on ice, then pelleted at 15,000 rcf for 2 minutes, resuspend 1100 µL sterile 0.85% NaCl-milli-Q and a 1/10 dilution series was made using 990 µL sterile 0.85% NaCl-milli-Q and 110 µL of library/previous dilution. 1000 µL portions of the dilutions up to and including 10^{-3} was plated on defined phosphorus media with 15 g/L Noble agar with 1 g/L glyphosate as sole phosphorus source, supplemented with Rif50, 150 mm petri-plates were used. This was grown for 6 days at 30°C, then 12 good colonies were selected and streaked on fresh plates of the same media. The 12 transconjugants were restreaked once to purify, and then tested in defined phosphorus liquid culture without phosphorus, with 1 g/L glyphosate, or 1 g/L equivalent ortho-phosphate. While every effort is made to eliminate contaminating phosphorus from the agar plates, growing in liquid culture is required as a final confirmation that the strains are growing using the glyphosate as the sole source of phosphorus rather than scavenged or stored phosphorus.

To confirm that restored glyphosate growth was due to the addition of the cosmid, rather than a mutation to the host genome, the cosmids were transferred to *E. coli* DH5α (NalR) through tri-parental conjugation using pRK600 as a helper plasmid. Conjugates were selected on LB-Nal30-Tet10, and restreaked once to purify. Controls (the individual strains

used in the triparental mating) were also streaked on LB-Nal30-Tet10 and no growth was found. The complementing cosmids were then moved back into *Agrobacterium* RIM-Id RfA *ΔphnGHIJ* using the same method and selection on TSA-Rf50-Tet10. The conjugates were again restreaked once to purify. The cosmids were isolated from *E. coli* DH5α using the geneJET miniprep kit from ThermoFisher. To test the diversity of these isolated plasmids, they were digested with HindIII and EcoRI and run on a 0.8% agarose gel. The three cosmids were found to have the same digest pattern. One cosmid was selected and sequenced by Illumina then assembled and annotated using BV-BRC using plasmidSPADES. The assembly produced multiple contigs, which were BLASTed against the pJC8 backbone sequence to assess completeness and purity. The contig with homology to pJC8 at both ends but not in the middle was selected as the fully assembled insert. The backbone sequences were trimmed from the insert fragment and this sequence was used for further analysis. Remaining contigs were found to map to *E. coli* and represent genomic contamination in the plasmid prep.

The trimmed insert was annotated using RAST through BV-BRC (Brettin et al., 2015; Olson et al., 2023). The trimmed insert was also BLASTed against the assembled RIM-Id genome (the source genome) (Z. Zhang et al., 2000).

4.2.8 Finding the minimal glyphosate necessary for growth

The glyphosate degrading *Agrobacterium* strains were originally isolated and tested on 1 g/L glyphosate. This was so that efficient glyphosate or phosphorus use would not be a limiting

factor for growth. However, strains did not consume a large portion of the available glyphosate. As such, the ELISA assay was unable to register significant differences in glyphosate levels after 4 days of growth from the initial value of 1 g/L glyphosate, even after dilution to within the glyphosate range specified by the manufacturer. To remedy this, a series of dilutions were made using defined phosphorus media with glyphosate, and RIM-Id RfA, GSA6-1, GSC6-1, and C58 RfB along with GSA6-1 were grown on 1 g/L, 0.75 g/L, 0.5 g/L, 0.25 g/L, 0.1 g/L, 0.05 g/L, 0.025 g/L, 0.01 g/L, 0.005 g/L, and 0 g/L glyphosate.

Inoculant was made by inoculating 5 mL of phosphorus free media (without any added phosphorus) with 20 µL of overnight TSB culture, and growing overnight at 30°C with shaking to exhaust stored phosphorus. 15 µL of overnight phosphorus-exhausted culture was used to inoculate 285 µL of the above concentrations of glyphosate in the defined phosphorus media. Growth was measured using the BioscreenC growth curve machine at 27°C with constant shaking (medium amplitude) for 120 h. This period of growth was expected to be beyond the stationary phase, so the growth curve at the various glyphosate concentrations was examined to find a shared point (96 hours) near the maximum growth level for the majority of the glyphosate concentrations.

4.2.9 Growth tests of *Agrobacterium* isolates, knockouts, and complements

Using the same defined media as before, the RIM-Id *phnGHIJ* knockout was re-tested, alongside the wildtype, and knockout with complementing cosmid pJC8-RIM-Id_3. Strains were tested in triplicate using 3 phosphorus conditions in the defined minimal media outlined

above. To minimize the effects of stored phosphorus, each culture was first grown in TSB liquid culture, then 20 µL of this culture was used to inoculate 5 mL of phosphorus free defined media and grown overnight at 30 ℃ with shaking. 15 µL of this phosphorus exhausted culture was used to inoculate 285 µL of one of 3 phosphorus conditions. Phosphorus conditions were: no added phosphorus, 0.025 g/L glyphosate, or 0.025 g/L glyphosate + 0.025 g/L (equivalent) $NaPO_4$. A negative control was performed in triplicate, inoculating with media without added phosphorus instead of culture. Growth was tested using a BioscreenC growth curve machine. Growth on glyphosate is shown here.

To confirm glyphosate degradation, glyphosate remaining in the glyphosate as sole source of phosphorus culture was quantified at the end point of the experiment and compared to the initial media. This was done using the ABRAXIS glyphosate ELISA and derivatization 96 well test kit from Gold Standard Diagnostics Horsham. The kit was used according to the manufacturer's specifications. Samples were diluted at 10^{-4} using 1/10 serial dilutions to bring samples within the quantification range specified by the kit (0 to 4 ppb (or ng/mL)). Dilution was done using milli-Q water, on ice, with 15 sections of vortexing on maximum speed between each dilution. A standard curve was created using the initial media with 0.025 g/L glyphosate, diluted with phosphorus free media. The standard curve was created at 0, 0.00625, 0.0125, 0.01875, and 0.025 g/L glyphosate, then diluted 10^{-4} using the same procedure as above.

Glyphosate was quantified according to the manufacturer's directions, by dividing the absorbance at that glyphosate concentration by the absorbance at 0 glyphosate. This

absorbance ratio was plotted against glyphosate concentration using excel on a logarithmic X-axis. The line of best fit was found and used to calculate the concentration of glyphosate remaining in each of the cultures containing glyphosate as the sole source of phosphorus.

4.2.10 C-P lyase operon comparison

The *phn* operon, implicated in glyphosate degradation, was annotated using RAST through BV-BRC and manually pulled from each strain for comparison (Brettin et al., 2015; Hove-Jensen et al., 2014; Olson et al., 2023). Genes within the C-P lyase operon were renamed to align with generally accepted naming conventions (Hove-Jensen et al., 2014).

4.3 Results

4.3.1 Genome sequencing, assembly, and annotation

The glyphosate soy enrichment cultures at enrichment number 6 provided 11 isolates that were sequenced by both Oxford Nanopore and Illumina NextSeq. These isolates were named for the culture they were isolated from (GSA6, glyphosate as phosphorus source, soy Soil as inoculant, subculture series A, subculture number 6) followed by a number (GSA6-1 is the first isolate from the GSA6 subculture) to create a unique code. Of these 11, 6 of them were fully assembled with no unresolved bridges. This included GSA6-1, GSA6-4, GSB6-1, GSB6-3, GSC6-1, and GSC6-2. The 5 that retained unresolved sections were dropped from further study. An example of the unresolved genome is shown for GSA6-3 (Figure 4.2).

Additionally, the glyphosate degrading *Agrobacterium* RIM-Id RfA and the glyphosate non-degrading C58 RfB were both fully assembled.

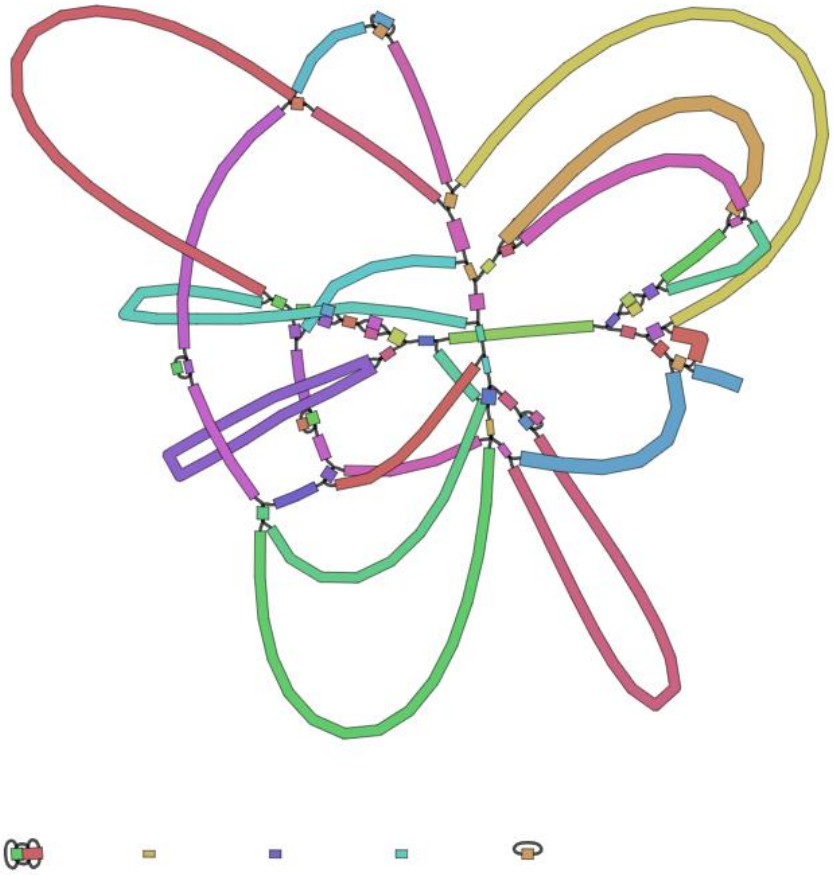


Figure 4.2 Unresolved Unicycler Assembly of GSA6-3: Unresolved bridges are shown as portions of DNA who's order and orientation are unclear, indicating incomplete assembly. Strains that were incompletely assembled were dropped from the study.

CheckM lineage_wf was performed to further confirm that resolved genomes were complete and had little contamination, results are shown in Table 4-3. All assemblies were

taxonomically assigned to the same group of Rhizobiaceae by CheckM. To compare completeness within this group, CheckM found that all 78 genomes in its reference set for Rhizobiaceae UID3554 share 840 single copy marker genes. These are genes that are expected to be present exactly once in all genomes of this genome set. The table displays the copy number actually found for each of the 840 predicted single copy genes. All genomes were found to contain exactly 1 copy of at least 830 of the 840 predicted single copy genes, and no genomes were found to have more than 2 copies of a predicted single copy gene.

Table 4-3 CheckM Genome Completeness Estimate

		Number of predicted single copy genes with *n* number of copies					
***Agrobacterium* Isolate**	**Number of Expected Single Copy Marker Genes**	**0**	**1**	**2**	**Completeness %**	**Contamination %**	**Strain heterogeneity**
RIM-Id	840	2	830	8	99.62	1.08	12.5
GSA6-1	840	1	837	2	99.96	0.38	0
GSA6-4	840	0	838	2	100	0.38	0
GSB6-1	840	0	838	2	100	0.38	0
GSB6-3	840	0	838	2	100	0.38	0
GSC6-1	840	0	838	2	100	0.24	0
GSC6-2	840	0	838	2	100	0.24	0
C58	840	0	840	0	100	0	0

All assemblies were taxonomically assigned to the same set of Rhizobiaceae genomes (UID3564) by CheckM. The set contained 78 genomes that share 840 single copy marker genes. These are genes that are expected to be present exactly once in all genomes of this genome set. The table displays the copy number actually found for each of the 840 predicted single copy genes. All genomes were found to contain exactly 1 copy of at least 830 of the 840 predicted single copy genes, and no genomes were found to have more than 2 copies of a predicted single copy gene. CheckM outputs the number of predicted genes with 3, 4, 5+ copies, but as 0 were found for any of these strains this was omitted from the table.

4.3.2 Isolate comparison

FastANI was used to calculate average nucleotide identity and provide an estimate of heterogeny between the fully assembled isolates. FastANI cuts the query genome into a set of non-overlapping fragments. The query fragments are then matched to the reference genome using Mashmap. Fragments may match to multiple places in the reference genome, and in this case the best match for each fragment is used to calculate the average nucleotide identity (Jain et al., 2018). In this way, genome rearrangements (due to biological differences or sequencing errors) have less effect on the nucleotide identity scores. Because the query is cut into fragments and compared to the reference, there are slight differences when comparing genome X to Y or Y to X.

FastANI (Table 4-4) showed that RIM-Id RfA is not closely related to any of the other isolates, having at least 11% difference in nucleotide identity. RIM-Id RfA is also most different from C58 RfB, despite the fact that C58 RfB and RIM-Id are the only two strains examined that have a Ti plasmid. Similarly, C58 RfB is at least 10% different from the other isolates. FastANI analysis also revealed that the isolates from the enrichment culture (GSA6-1, GSA6-4, GSB6-1, GSB6-3, GSC6-1, GSC6-2) are very closely related to each other. However, these isolates do appear to fall into two groups. GSA6-1, GSA6-4, GSB6-1, and GSB6-3 are all within 99.9% identical to one another, and represent the same strain. By comparison, this group is only about 98.3% identical to GSC6-1 and GSC6-2, while GSC6-1 and GSC6-2 are more than 99.99% identical to each other. By comparing average nucleotide identity of the fully assembled isolates, GSA6-1 and GSC6-1 are left as two representative

strains of the same species, and RIM-Id RfA and C58 RfB as being two more distinct species. Despite being slightly less complete according to CheckM results, GSA6-1 was still selected as a representative genome of the GSA6-1, GSA6-4, GSB6-1, and GSB6-3 group because previous work had already been performed with it, including making a genomic library. Missing one predicted single copy gene was considered acceptable.

Table 4-4 FastANI Estimate of Average Nucleotide Identity

		Query Genome							
		GSA6-1	**GSA6-4**	**GSB6-1**	**GSB6-3**	**GSC6-1**	**GSC6-2**	**RIM-Id RfA**	**C58 RfB**
Reference Genome	**GSA6-1**	100	99.9959	99.9768	99.9778	98.2988	98.2993	88.5893	89.3847
	GSA6-4	99.9943	100	99.9723	99.9735	98.2938	98.2948	88.5978	89.4388
	GSB6-1	99.9629	99.9685	100	99.9987	98.3288	98.3230	88.5208	89.3892
	GSB6-3	99.9608	99.9575	99.9986	100	98.2816	98.2822	88.5695	89.3564
	GSC6-1	98.2788	98.2942	98.3158	98.2753	100	99.9995	88.5473	89.2540
	GSC6-2	98.2801	98.2953	98.3160	98.2765	99.9993	100	88.5567	89.2611
	RIM-Id	88.5242	88.5147	88.6074	88.6028	88.5208	88.5301	100	87.2853
	C58 RfB	89.3349	89.2980	89.4161	89.3970	89.2832	89.2855	87.4511	100

FastANI was used to compare the average nucleotide identity between sequenced strains. FastANI returns the percent identity between the query and the reference genome, using non-overlapping fragments to minimize the effects of genome rearrangements and incomplete sequencing. C58 RfB, and RIM-Id RfA were found to be both different from each other and from the isolates from the enrichment cultures (GS-). The enrichment culture isolates were found to all be closely related and likely the same species, but were separated into two distinct strains. With GSA6-1, GSA6-4, GSB6-1, and GSB6-3 being members one strain (highlighted), and GSC6-1 and GSC6-2 being members of the other (highlighted).

Assembled isolates were found to contain a circular chromosome, a linear chromid, and a plasmid, and in the case of RIM-Id RfA and C58 RfB, an oncogenic Ti plasmid was found. When the Ti plasmid is included, this is also the structure found in previous sequencing projects for *Agrobacterium* C58 (Nester, 2015). A circular chromosome along with a linear chromid is also the accepted structure of biovar-1 *Agrobacterium* species generally (Weisberg et al., 2023). Assembly reports were produced by BV-BRC's genome assembly program, and taken from their assembly report, shown in Figure 4.3 (Olson et al., 2023). These isolates were resolved without bridges, and circular elements were able to assemble without gaps.

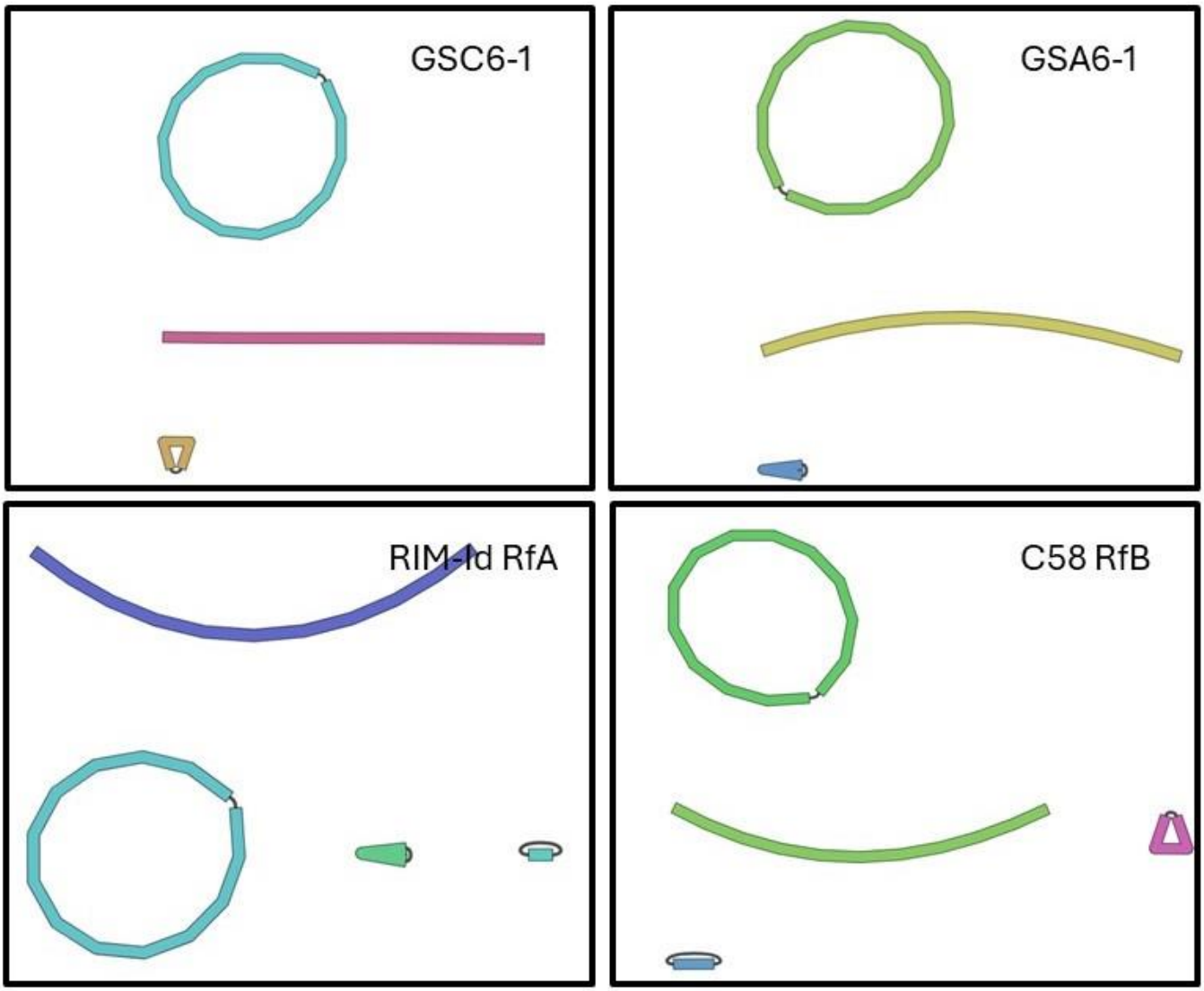


Figure 4.3 Genome Assembly Report: The main chromosome, as well as either one or two plasmids were able to fully circularize, and the chromid was able to assemble without gaps. Adapted from Bacterial-Viral Bioinformatics Resource Center **(Olson et al., 2023)**

ProgressiveMauve was used to align the unique genomes. Few rearrangements are seen in the circular chromosome, which includes the C-P lyase genes (Figure 4.4). Significant rearrangements are seen in the linear chromid, along with the oncogenic and non-oncogenic plasmids, with greater changes between RIM-Id, GSA6-1/GSC6-1, and C58 RfB than between GSA6-1 and GSC6-1. This fits with the expectation that GSA6-1 and GSC6-1 are strains of the same species as shown strain identification and by average nucleotide identity. Fragments of the non-oncogenic plasmid in GSA6-1, GSC6-1, and C58 RfB are found in the chromid of RIM-Id RfA, and portions of the non-oncogenic plasmid in RIM-Id RfA are found in the chromid of GSA6-1, GSC6-1 and C58 RfB. Despite the fact that RIM-Id RfA and C58 RfB are both roughly equally distantly related from GSA6-1/GSC6-1 and from each other, the genome arrangement of C58 RfB appears similar to GSA6-1 and GSC6-1 and clearly divergent from RIM-Id RfA. There are no oncogenic plasmids in the *A. tumefaciens* GSA6-1 or GSC6-1 strains studied here. The genomes of these isolates are accessible through an NCBI BioProject, accession PRJNA1198028.

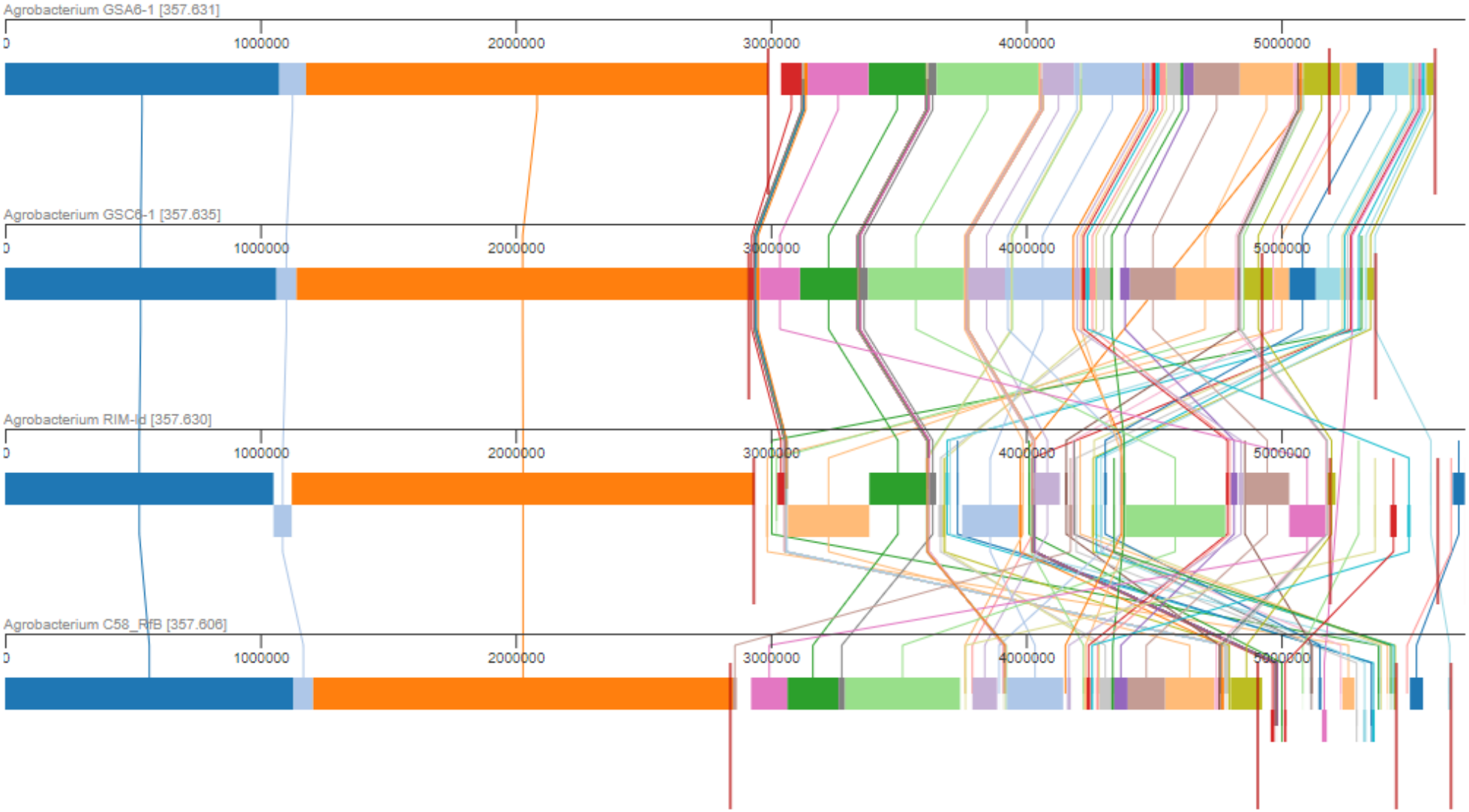


Figure 4.4 ProgressiveMauve Genome Alignment: ProgressiveMauve shows genome rearrangement between our sequenced *Agrobacterium* strains. Little rearrangement is seen between the chromosomes, however significant rearrangement is seen within and between the chromid, and oncogenic and non-oncogenic plasmids. There are no oncogenic plasmids in the *A. tumefaciens* GSA6-1 or GSC6-1 strains studied here. The genomes of these isolates are accessible through an NCBI BioProject, accession PRJNA1198028.

4.3.3 Strain identification by GTDB-Tk

Genome Taxonomy Database (GTDB-Tk) uses two methods to identify strains, closest strain based on highest average nucleotide identity to a set of reference genomes, and placement in a tree of reference genomes (Chaumeil et al., 2020). For all strains presented here, both average nucleotide identity and closest neighbor identified the same taxonomy (Table 4-5). All species are *Agrobacterium*, with those isolated from the enrichment culture being *Agrobacterium tumefaciens.* The strain RIM-Id RfA, was identified as *Agrobacterium pusense*, and C58 RfB was identified as *Agrobacterium fabrum*.

Table 4-5 Strain Identification by Genome Taxonomy Database

Genome	FastANI Closest Taxonomy (Reference Genome)	ANI of Genome to FastANI Taxonomy	Closest Neighbor Taxonomy (Reference Genome)	ANI with Closest Neighbor
GSA6-1	*Agrobacterium tumefaciens* (GCF_001541315.1)	97.62 %	*Agrobacterium tumefaciens* (GCF_001541315.1)	97.62 %
GSC6-1	*Agrobacterium tumefaciens* (GCF_001541315.1)	97.84 %	*Agrobacterium tumefaciens* (GCF_001541315.1)	97.84 %
RIM-Id RfA	*Agrobacterium pusense* (GCF_900102105.1)	99 %	*Agrobacterium pusense*	99 %
C58 RfB	*Agrobacterium fabrum* (GCF_000092025.1)	100 %	*Agrobacterium fabrum*	100 %

Genome Taxonomy Database took kit (GTDB-tk) identified all strains using both average nucleotide identity and closest neighbor reference. In all cases both of these methods produced the same estimated species. GSA6-1 and GSC6-1 were both identified as *Agrobacterium tumefaciens*, while RIM-Id RfA was identified as *Agrobacterium pusense* and C58 RfB was identified as *Agrobacterium fabrum*.

4.3.4 Knockout of *phnGHIJ* in *Agrobacterium pusense* RIM-Id RfA

Based on antibiotic resistance (kanamycin sensitive, spectinomycin resistant), two likely identical knockouts were produced in *A. pusense* RIM-Id RfA. To further test for a successful knockout of *phnGHIJ* from *A. pusense* RIM-Id RfA, a PCR was performed using two primer sets. One pair was situated outside of the knockout region (and outside of the overlap regions used to create the knockout) and would amplify a 1789 bp band only if the knockout was successful. The second primer set uses a forward primer within the knockout, and the reverse primer from the first set. This would amplify only when the *phn* operon is still intact. Both of the knockouts and the wildtype showed the expected pattern (Figure 4.5). Knockout number 2 was selected for future work, but both were saved in our library of strains.

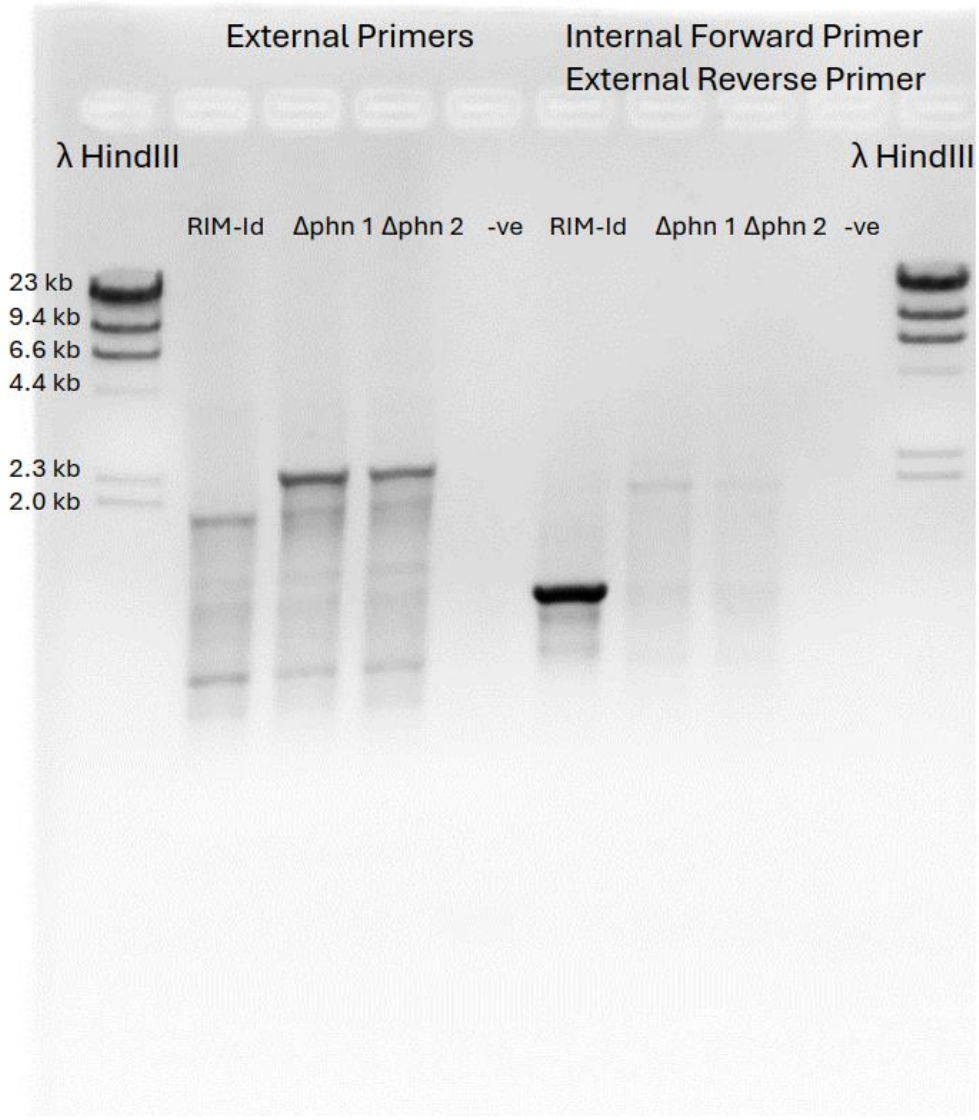


Figure 4.5 *phnGHIJ* Knockout PCR Confirmation: PCR was used to confirm the knockout. The knockouts (Δphn1, Δohn2) produce a 1789 bp band when primers outside and facing into the knockout are used. This band appears slightly high relative to the ladder but this is likely an artefact of the gel. This band is not present when DNA from the wild type is used. A second test, using a forward primer that binds within the removed region in the knockout and the reverse primer from the first pair, amplifies a 1074 bp band only in the wild type, but not in the knockout as the forward primer is unable to bind. Other bands indicate non-specific binding within the 5.7 Mbp genome and do not differ significantly between the wild type or the knockouts.

4.3.5 Genomic library

The backbone used to make the library was fully cut, producing 0 colonies from a 50 ng transformation test, and almost fully phosphorylated, producing 7 colonies from a 50 ng religation test. In contrast, 50 ng of uncut pJC8 backbone produced ~800 colonies. The RIM-Id RfA library produced approximately 450,000 clones and the GSA6-1 library produced approximately 360,000 clones. 12 clones from each library were grown, isolated, and digested using HindIII and EcoRI, alongside pJC8 backbone. These clones were compared to each other for identical patterns or missing digests within the same library (Figure 4.6). In the RIM-Id library, clones 1 and 8; and 2 and 12 look similar, but have a different band pattern below 2 kb and are in fact unique. One clone (4) does not appear to contain any band pattern, possibly due to an extraction, digest, or loading error or it is a tetracycline resistant mutant of the *E. coli* HB101 host that does not contain a cosmid. This leaves 11 of 12 tested hosts containing clones in the library derived from RIM-Id. In the library derived from GSA6-1, clones 3 and 6 may be identical, and clone 10 appears not to have any bands, again possibly due to experimental error or due to a mutant host strain. This leaves 10/12 clones unique and present within the library derived from GSA6-1.

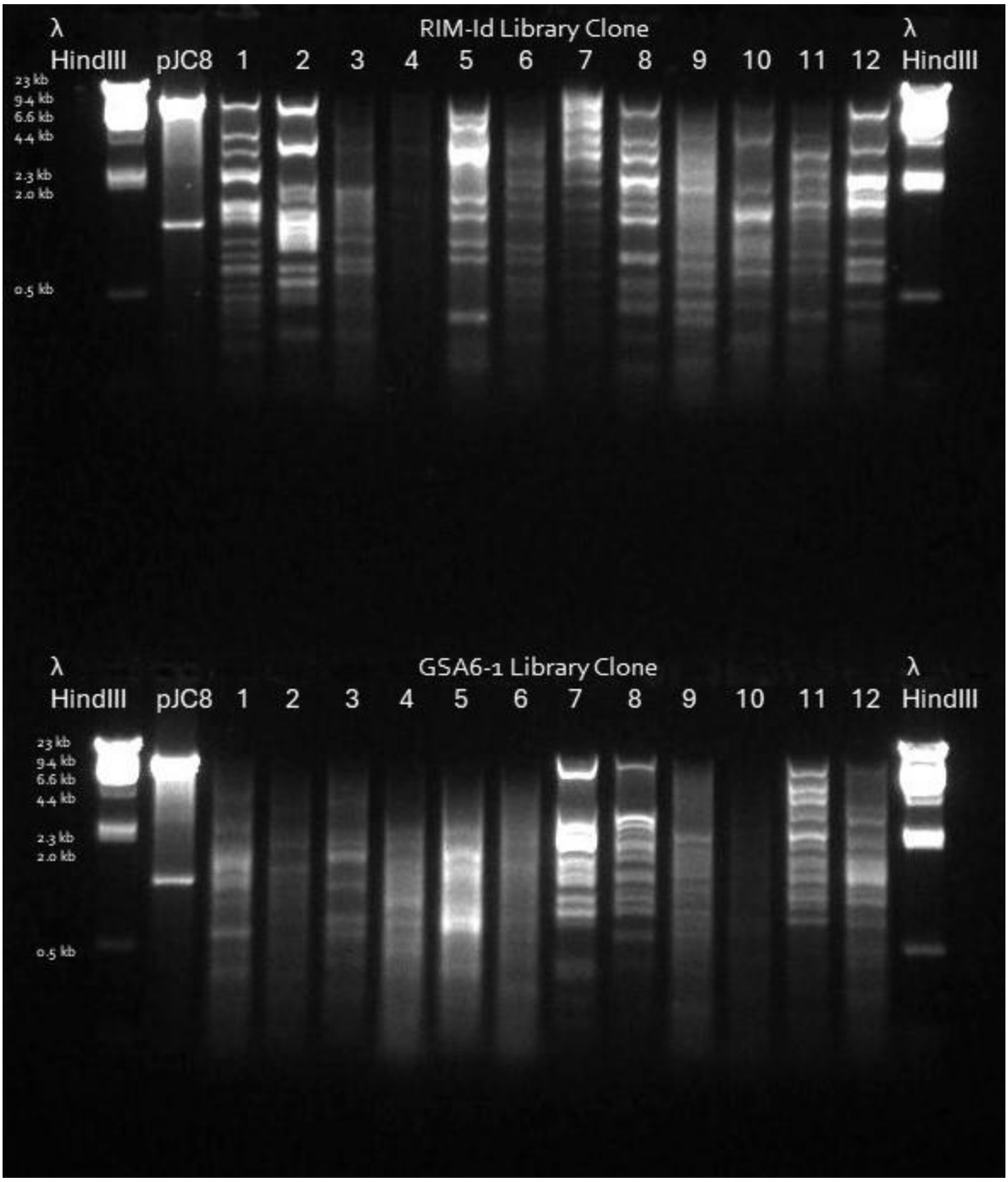


Figure 4.6 Genomic Library Clones: Of the RIM-Id library, clones 1 and 8; and 2 and 12 look similar, but have a different band pattern below 2 kb and are in fact unique. Clone 4 also appears not to have digested, this could have been an extraction, loading, or digest error, or clone 4 may not contain a cosmid. Of the RIM-Id library, 11 of the tested isolates had unique clones. For the GSA6-1 library, 3 and 6 may be identical, and clone 10 may be an extraction, loading, or digest error or may not contain a cosmid. This leaves a GSA6-1 library with 10 of the 12 tested clones being unique and containing a cosmid.

4.3.6 Glyphosate degrading cosmid complementation

Of the 12 transconjugants selected from the defined phosphorus media with 15 g/L Noble agar, 1 g/L glyphosate, and 50 mg/L rifampicin, none grew without any phosphorus source (negative control), all grew when ortho-phosphate was provided, and 5 grew on glyphosate as the sole source of phosphorus after 4 days at 30°C. 3 of the 5 were successfully transferred to *E. coli* DH5α and back to *Agrobacterium* RIM-Id RfA *ΔphnGHIJ*, where they again restored growth on glyphosate. When these three cosmids were digested with HindIII and EcoRI and run on a 0.8% agarose gel (Figure 4.7) they showed the same digestion pattern so they were determined to likely be replicates of the same cloning event.

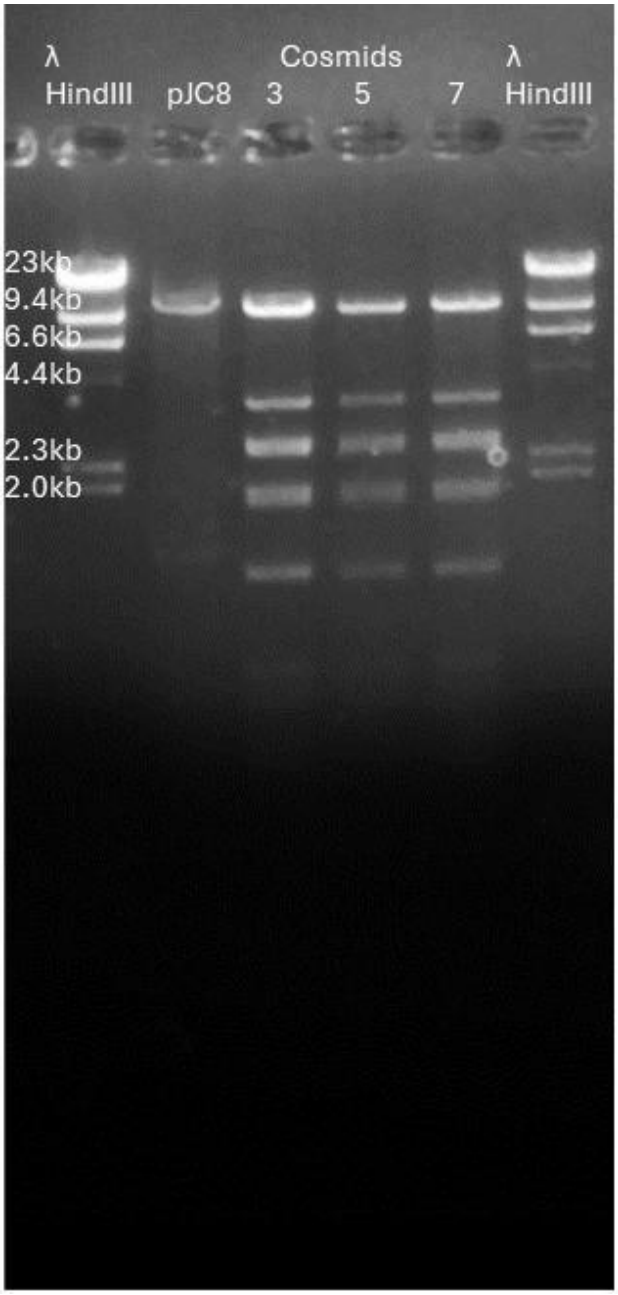


Figure 4.7 Glyphosate Degradation Restoring Cosmids: 3 cosmids that restored growth on glyphosate as sole phosphorus. All three cosmids (3, 5, and 7) produced the same band pattern when digested using HindIII and EcoRI, and therefore likely hold the same genomic fragment.

Because the agarose gel showed all three cosmids to be the same, only cosmid 3 (pJC8-RIM-Id_3) was sequenced. The entire trimmed insert region was found by BLAST to match perfectly to the source genome (*A. pusense* RIM-Id RfA). The annotation (Figure 4.8) revealed that it contained *phnFGHIJKLO*, along with a 10 kbp region upstream of the phn fragment. The complementing cosmid did reveal a replacement of a large portion of the phn complex, rather than just the *phnGHIJ* that was knocked out in the host strain.

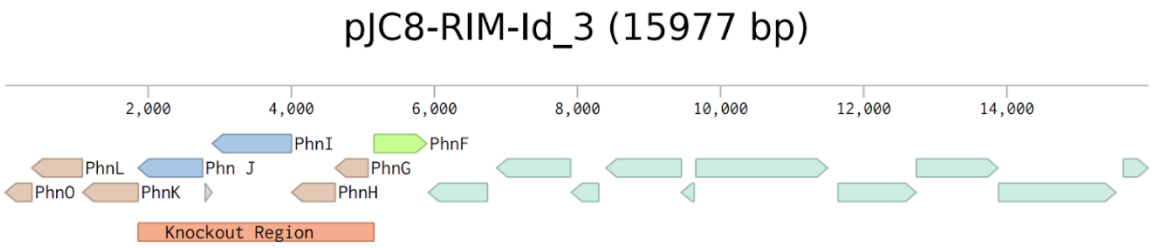


Figure 4.8 Glyphosate Degradation Restoring Cosmid Gene Annotation: Cosmid 3 from above was renamed pJC8-RIM-Id_3. It was shown to contain *phnFGHIJKLO* and was an exact match to the genome of RIM-Id RfA that it came from. Though it was selected in a knockout of *phnGHIJ*, additional components of the *phn* operon were found in the cosmid, along with a number of upstream genes.

4.3.7 Minimal glyphosate required for growth

At 96 hours, most cultures had an OD600 at or very close to their maximum, so this was selected as a standardized point to compare growth on various glyphosate concentrations. It was found that at less than 0.025 g/L glyphosate, optical density at the stationary phase drops off precipitously (Figure 4.9). Growth and control cultures were tested in triplicate, averaged, and the average optical density of the control at the same glyphosate concentration was

subtracted. Error bars show the highest and lowest measurements of the triplicate. For clarity, samples well in excess of limiting glyphosate are omitted form this graph, however the full graph can be found in Appendix B, Figure A.1 plus and minus 1 standard deviation based on the triplicates. Based on this, further glyphosate degradation tests were performed using a starting concentration of 0.025 g/L glyphosate.

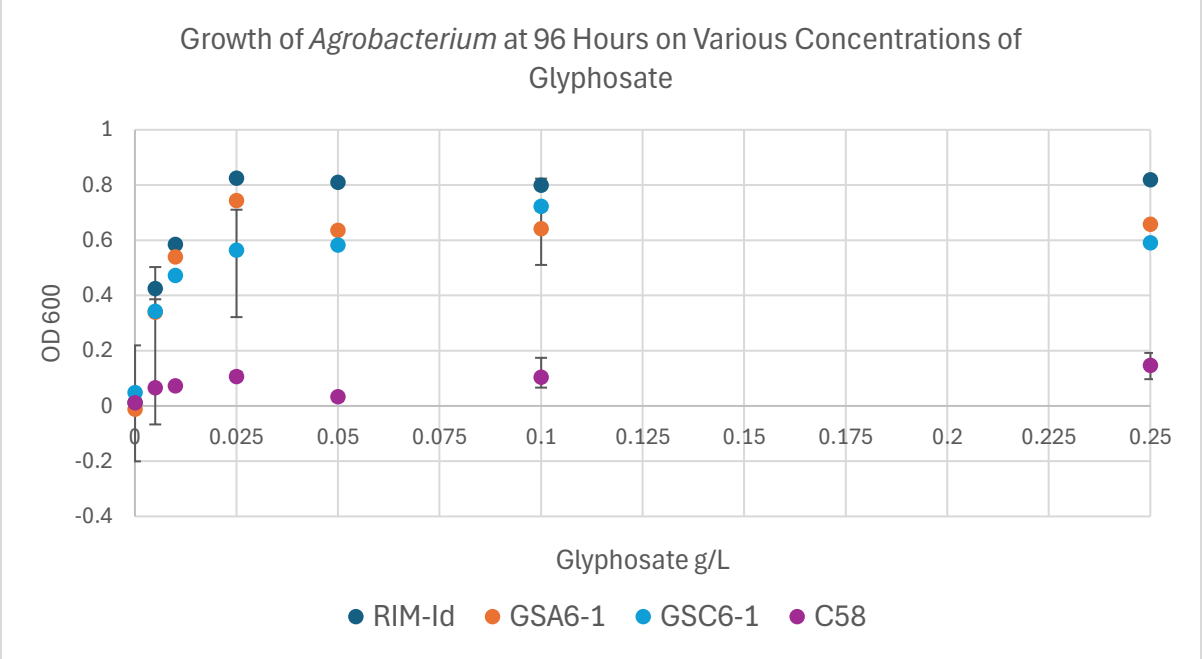


Figure 4.9 Maximum Growth on Various Glyphosate Concentrations: Maximum growth was reached at roughly 96 hours post inoculation. Using the optical density at this point, the concentration at which maximum growth is decreased can be found. This was found to be at less than 0.025 g/L glyphosate, with 0.025 g/L still allowing for good growth. Growth was done in triplicate for each species and each condition. Error bars show the maximum and minimum optical density (after taking zeroing into account). Only growth from 0 to 0.25 g/L is shown for clarity.

4.3.8 Growth test of RIM-Id, *ΔphnGHIJ*, pJC8-RIM-Id-3

When grown on defined phosphorus media with 0.025 g/L glyphosate as the sole source of phosphorus, wild type *A. tumefaciens* RIM-Id RfA was able to grow well and metabolize the glyphosate. By knocking out the C-P lyase genes *phnGHIJ*, RIM-Id RfA lost the ability to grow on glyphosate as a sole source of phosphorus, but retained its ability to grow when also provided with ortho-phosphate (Appendix B, figure B.2, B.3, and B.4). Growth was restored by providing the strain with the cosmid pJC8-RIM-Id-3, isolated from the RIM-Id genomic library and containing a portion of the *phn* operon (*phnFGHIJKLO*). Growth on glyphosate as sole source of phosphorus is shown in Figure 4.10. Positive (glyphosate and ortho-phosphate) control and negative (no added phosphorus source) control were as expected for all strains.

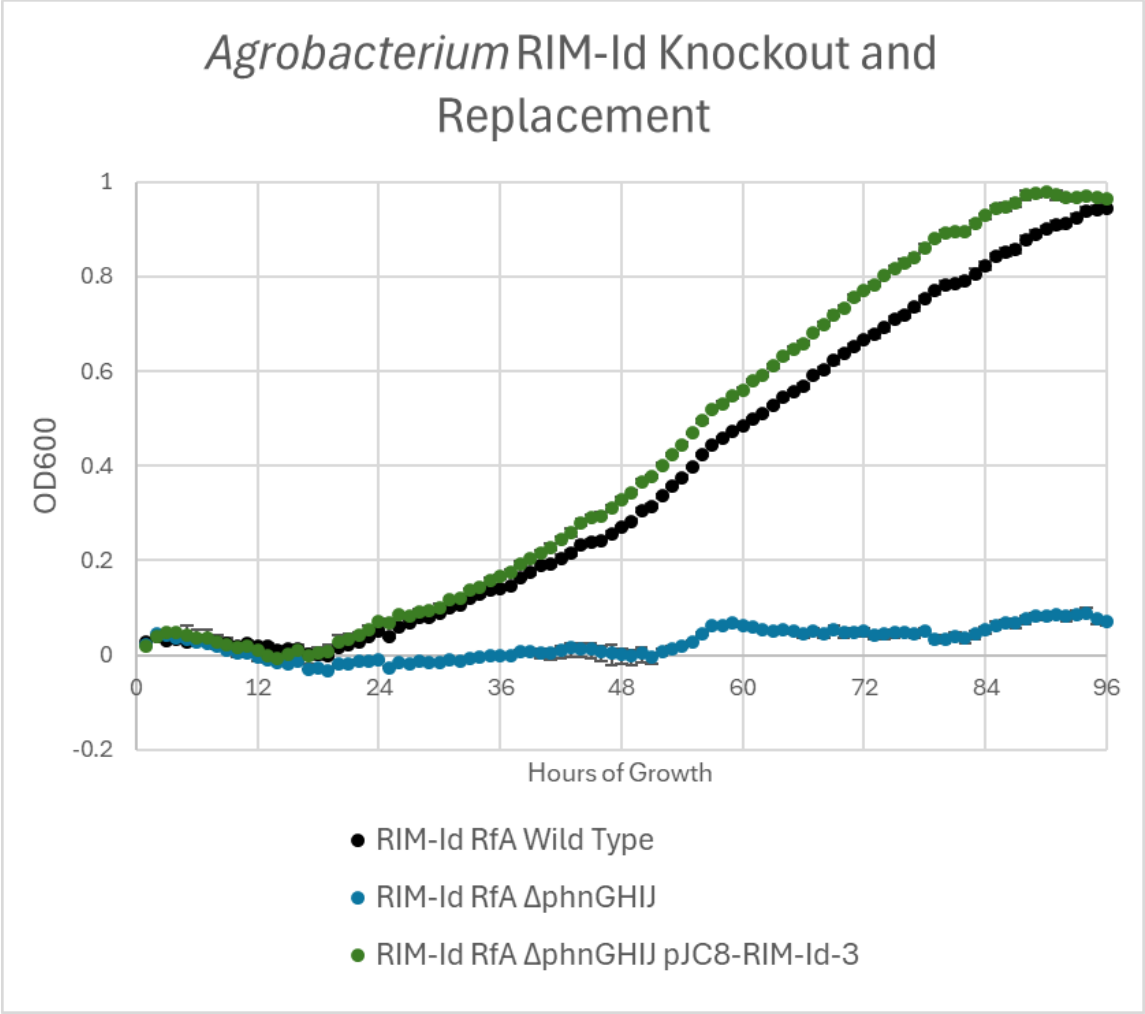


Figure 4.10 Agrobacterium RIM-Id Knockout and Replacement: *A. tumefaciens* RIM-Id RfA is able to grow well on glyphosate as the sole source of phosphorus. Growth is prevented when the C-P lyase genes *phnGHIJ* are knocked out. Growth is restored in the knockout when the genes are added back as part of a cosmid isolated from the RIM-Id genomic library. Three replicates of each condition were zeroed by subtracting the average optical density of the sterile control. Points represent the average absorbance and error bars show the highest and lowest measurements.

After growing for 96 hours, ELISA was performed to quantify the remaining glyphosate (Figure 4.11). The same batch of media at an initial glyphosate concentration of 0.025 g/L was used for all of the tests and to create the standard curve. After 96 hours, the

sterile uninoculated control, showed a slight increase in measured glyphosate concentration, possibly due to evaporation concentrating the media, inaccuracies in the test or dilution, or a combination of both. The wild type *A. pusense* RIM-Id RfA showed no remaining glyphosate, while the *phnGHIJ* knockout showed little glyphosate degradation. The cosmid pJC8-RIM-Id-3 was able to rescue the knockout phenotype and allow for degradation of the entire 0.025 g/L of glyphosate.

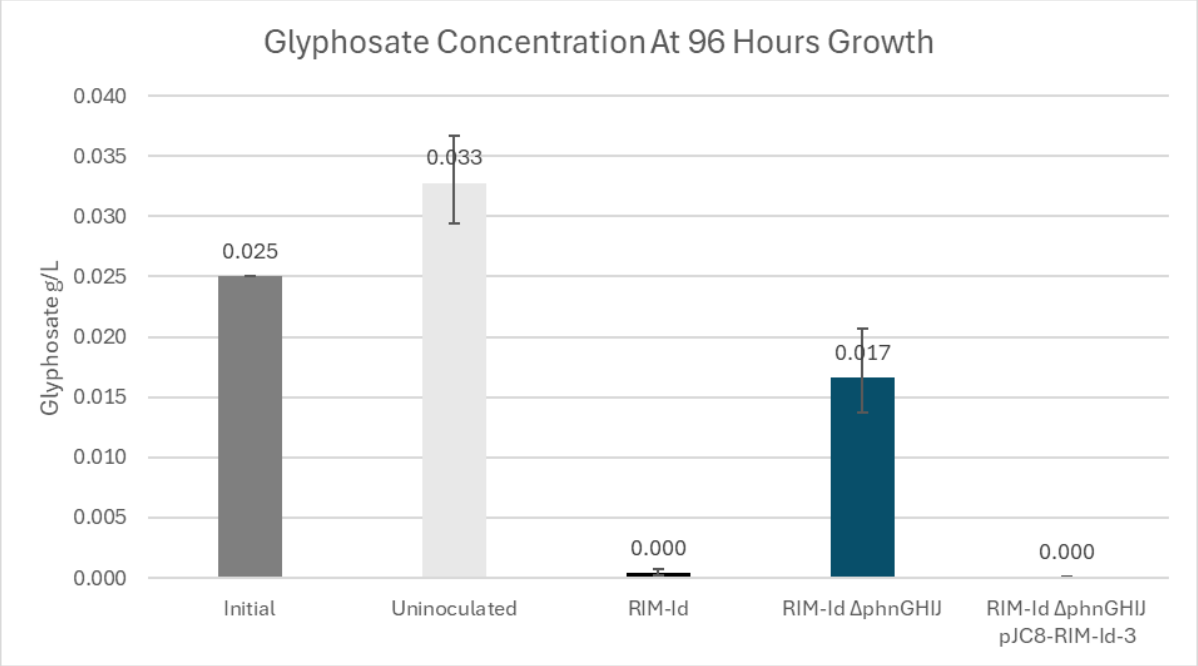


Figure 4.11 Glyphosate Concentration At 96 Hours Growth: Remaining glyphosate was quantified by ELISA. The wild type *A. pusense* RIM-Id RfA was able to degrade the entire 0.025 g/L of glyphosate, while the knockout of the C-P lyase genes phnGHIJ was unable to degrade significant glyphosate. The cosmid pJC8-RIM-Id-3 restored glyphosate degradation. Bars show the average glyphosate remaining (of the triplicate cultures), and error bars show the highest and lowest measured glyphosate for that treatment. For comparison, initial glyphosate was 0.025 g/L and was not measured by ELISA, but is set at the initial 0.025 g/L glyphosate of the media used to make the standard curve and used for all growth tests.

4.3.9 Growth tests of wild type *Agrobacterium*, glyphosate quantification after growth by ELISA

Both strains of *A. tumefaciens* GSA6-1 and GSC6-1 were able to grow well on 0.025 g/L glyphosate as the sole source of phosphorus (Figure 4.12). Though these strains were isolated from a glyphosate enrichment culture and had the opportunity to evolve efficient growth on glyphosate as the sole source of phosphorus, neither of them grow as well as *A. pusense* RIM-Id RfA, which was not isolated for its glyphosate degradation ability. *A. fabrum* C58 RfB can also grow on glyphosate as a sole source of phosphorus, but not as well as either the *A. pusense* RIM-Id RfA or *A. tumefaciens* GSA6-1 and GSC6-1 strains. *A. fabrum* C58 RfB also exhibits a long lag time relative to *A. tumefaciens* GSA6-1 and GSC6-1, and *A. pusense* RIM-Id RfA, despite the fact that all strains in this study were primed for growth on alternative phosphorus sources by first undergoing a phosphorus exhaustion step. None of these strains grew well on the media without a source of phosphorus, and all strains grew faster but to a similar respective optical density on media with ortho-phosphate as well as glyphosate as they did on glyphosate as sole source of phosphorus (Appendix B, Figure B.5 and B.6).

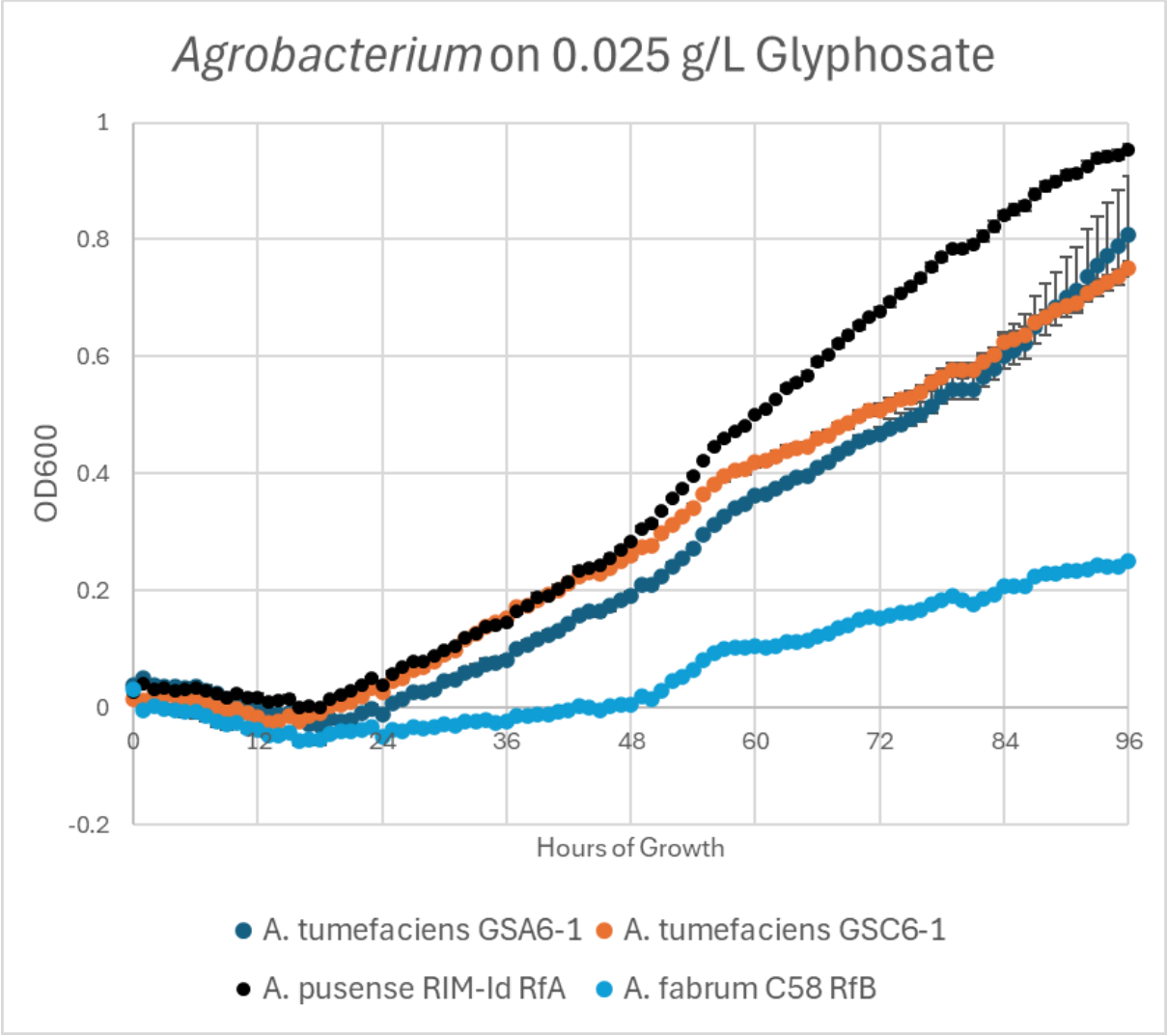


Figure 4.12 *Agrobacterium* Growth on 0.025 g/L Glyphosate: *A. tumefaciens* GSA6-1 and GSC6-1 both grow well on glyphosate as a sole source of phosphorus, but not as well as *A. pusense* RIM-Id RfA. *A. fabrum* C58 RfB grows on glyphosate, but does not grow well under these conditions. Three replicates of each condition were zeroed by subtracting the average optical density of the sterile control. Points represent the average absorbance and error bars show the highest and lowest measurements.

An ELISA assay was used to quantify the remaining glyphosate after growth (Figure 4.13). GSA6-1, RIM-Id, and C58 were all able to fully degrade the initial 0.025 g/L

glyphosate, while GSC6-1 was able to degrade 0.020 g/L in 96 hours. After 96 hours, the sterile uninoculated control, showed a slight increase in measured glyphosate concentration, possibly due to evaporation concentrating the media, or inaccuracies in the test or dilution, or a combination of both. The bar showing initial glyphosate shows the predicted 0.025 g/L glyphosate of the starting culture, and does not represent an actual measurement. The 0.025 g/L sample is included for comparisons sake. The media used for these growth tests was also used to create the standard curve in the ELISA plate.

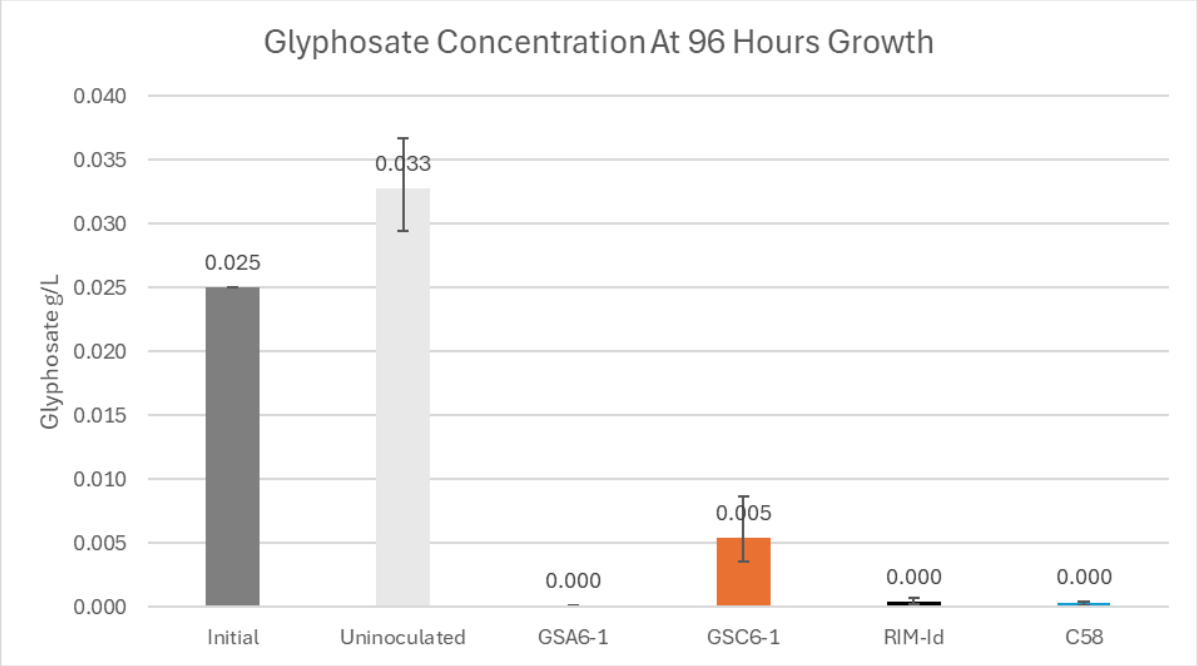


Figure 4.13: Remaining Glyphosate Concentration at 96 Hours Growth: Remaining glyphosate was quantified by ELISA. All strains degraded the majority of the glyphosate, with GSA6-1, RIM-Id, and C58 degrading all of the 0.025 g/L initial glyphosate within 96 hours. GSC6-1 was able to degrade most of the glyphosate, with only 0.005 g/L remaining. Bars show the average glyphosate remaining (based on triplicate cultures), and error bars show the highest and lowest measured glyphosate for that treatment. The bar showing initial glyphosate was not measured by ELISA, but is set at the initial 0.025 g/L glyphosate of the media for comparison. The same media was used to make the standard curve and used for all growth tests.

4.3.10 Phn multiple sequence alignment

RIM-Id, GSA6-1, GSC6-1, and C58 RfB all appear to have the full complement of phn genes (Figure 4.14), with the exception of the second *phnM* found in *Agrobacterium radiobacter* (Hove-Jensen et al., 2014). The lack of a second *phnM* was confirmed by BLAST, the *phnM* sequence was found to only match one location in the genomes of each of the organisms. All strains were missing *phnP* but contained *duf1045*, in agreement with previously researched *Agrobacterium* (Hove-Jensen et al., 2014; Masotti et al., 2021).

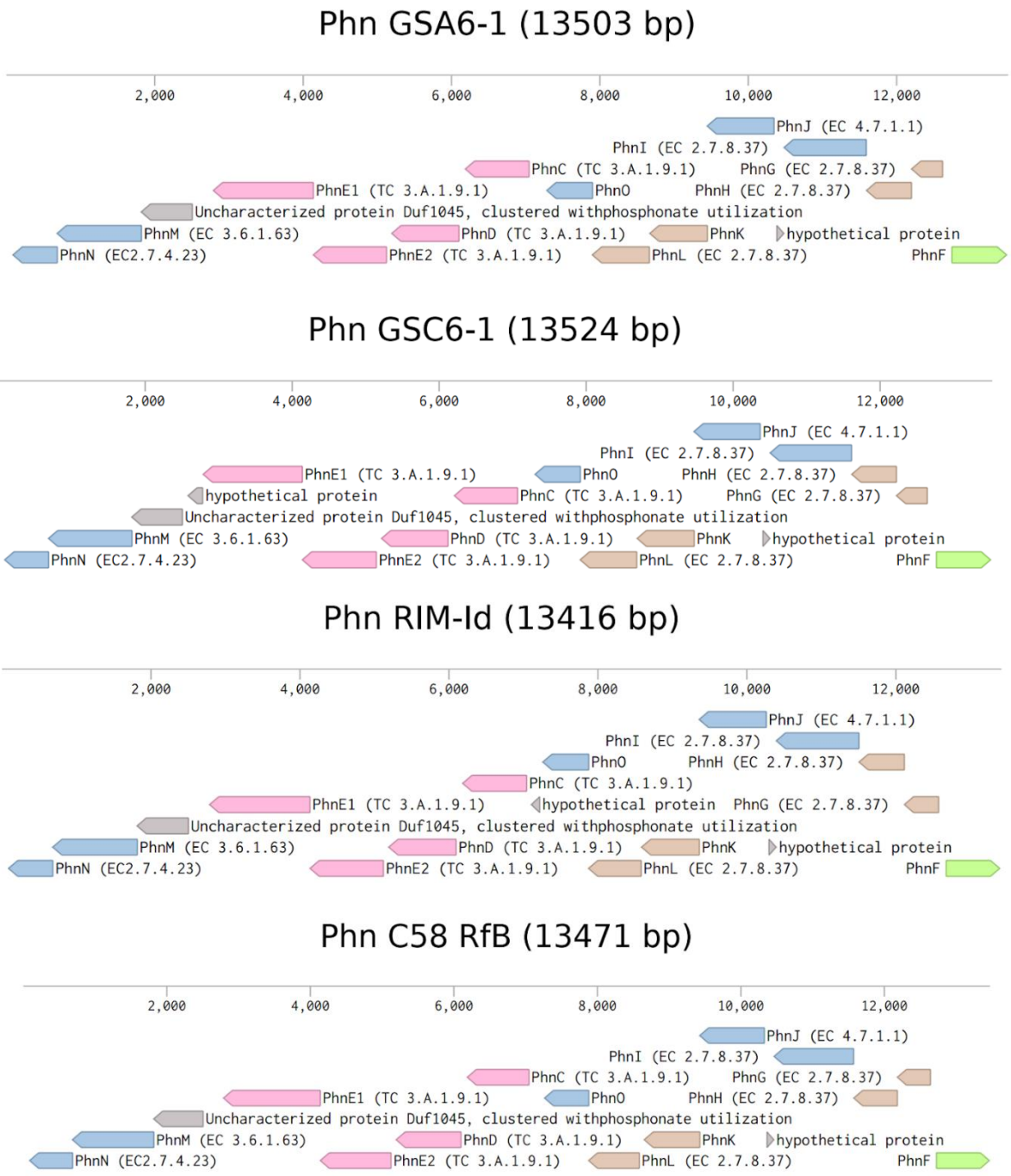


Figure 4.14 Phn Operons in *Agrobacterium*: All strains contained the full complement of expected *phn* genes for an *Agrobacterium*, with the exception that none of them contained a second *phnM*. All strains were missing *phnP* but contained *duf1045*.

4.4 Discussion

4.4.1 Genome sequencing and strain identification

The genomes of 4 isolates of *Agrobacterium*, including two strains of *A. tumefaciens*, a strain of *A. pusense*, and an *A. fabrum*, were successfully sequenced and assembled. The genomes of these isolates are accessible through an NCBI BioProject, accession PRJNA1198028. CheckM found that all strains were 99.96-100% complete, with few missing expected single copy genes and no single copy genes with more than 2 copies when compared to an automatically selected marker set built from multiple genomes within the Rhizobiacea family (Parks et al., 2015). The CheckM score for RIM-Id was lowest, with a strain heterogeny score of 12.5, this is likely an artifact of strain handling, as RIM-Id was sequenced in two batches from two DNA isolations performed years apart. Long read sequencing was done using PacBio for RIM-Id, while all other isolates were sequenced with Oxford Nanopore. All strains also underwent short read sequencing using Illumina. Despite this small amount of heterogeny, all genomes presented here are complete and correct as evidence by their completeness score.

Average nucleotide identity found that all isolates from the enrichment cultures (those starting with GS-) were of the same species and fell into two broader groups. GSA6-1, GSA6-4 GSA6-1, GSB6-1, and GSB6-3 are all within 99.9% identical to one another and GSA6-1 was selected as the representative isolate for futher testing. GSC6-1 was selected as the representative of GSC6-1 and GSC6-2 which are also 99.9% identical to each other. The

GSA6-1 and GSC6-1 groups were found to be about 98.3% identical to each other. Both of these were found to be about 89% identical to RIM-Id and C58 RfB, which are themselves only about 87% identical to each other. GTDB uses both average nucleotide identity and a closest neighbor tree to identify the most likely organism (Chaumeil et al., 2020). In all cases, both of these estimates agreed giving us a high degree of confidence that GSA6-1 and GSC6-1 are both *Agrobacterium tumefaciens,* RIM-Id RfA is *Agrobacterium pusense,* and C58 RfB is *Agrobacterium fabrum*. C58 is a model organism (the first *Agrobacterium* to be sequenced) and was expected to be identified as *Agrobacterium fabrum* (Weisberg et al., 2023; Wood et al., 2001).

All genomes consisted of a large circular chromosome, a smaller linear element (chromid), and an additional plasmid that does not harbor any *vir* genes. In addition, *Agrobacterium pusense* RIM-Id and *Agrobacterium fabrum* both contained a Ti plasmid containing virulence (*vir*) genes. This genome arrangement (circular chromosome, large linear chromid, various oncogenic and non-oncogenic plasmids) is consistent with true *Agrobacterium* species (biovar 1 and biovar 1-like) (Weisberg et al., 2023). Significant rearrangement within and between both the chromid and the non-oncogenic plasmid is seen in the progressiveMAUVE analysis. It appears that multiple fragments of the non-oncogenic plasmid in *A. tumefaciens* GSA6-1 and GSC6-1, and *A. fabrum* C58 RfB are instead located in the chromid of *A. pusense* RIM-Id RfA. This adds additional validity to the hypothesis by Weisberg et al in 2022 that rearrangement between and within the less conserved replicons (the chromid and oncogenic & non-oncogenic plasmids) generate variability that is

fundamental to the robustness of *Agrobacterium* in the environment (Weisberg et al., 2022). The C-P lyase operon is located on the more conserved circular chromosome in all cases studied here, so the additional genome rearrangement is likely unrelated to glyphosate degradation. Genome alignment appears to show that *A. fabrum* C58 RfB has a similar genome structure to *A. tumefaciens* GSA6-1 and GSC6-1, and all three have a more divergent genome structure from glyphosate degrading *A. pusense* RIM-Id RfA. This is despite the fact that *A. fabrum* grows poorly in the media used in this experiment, while *A. pusense* RIM-Id RfA and both strains of *A. tumefaciens* grow well. Average nucleotide identity, which only penalizes rearrangement when it splits a 1000 bp query sequence, shows all three species to be about 10% different from each other despite the rearrangements, and shows the two strains of *A. tumefaciens* to be only about 2% divergent from each other.

4.4.2 Genomic Library

Genomic expression libraries were successfully made from the glyphosate degrading *Agrobacterium* RIM-Id and GSA6-1. The RIM-Id library contained approximately 450,000 clones and the 11 of the 12 tested clones were found to contain cosmids with unique restriction digest patterns. The GSA6-1 library contained approximately 360,000 clones and 10 of the 12 tested clones were shown to contain cosmids with unique restriction digest patterns. Unique restriction digest patterns suggest unique inserts within those cosmids. Assuming even distribution and an average insert size of 40,000 bp, this corresponds to a coverage of approximately 2800X over the 5.7 Mbp genome of RIM-Id, and 2000X coverage

over the 5.6 Mbp genome of GSA6-1. The high quality and purity of the isolated genomic DNA allowed these libraries to have the large size and coverage that they did.

The RIM-Id library was also further confirmed by transferring to a strain of *A. pusense* RIM-Id RfA Δ*phnGHIJ*, and was shown to restore glyphosate degradation (below). This confirms that these libraries are able to express their genes and should be able to transfer into related organisms, especially those in the Rhizobiaceae family, that are unable to degrade glyphosate. Due to time constraints, these libraries were not able to confirm cross-functionality of the *A. tumefaciens* GSA6-1 library in *A. pusense* RIM-Id Δ*phnGHIJ*.

4.4.3 A knockout shows that *Agrobacterium pusense* RIM-Id RfA requires C-P lyase to degrade glyphosate

The C-P lyase knockout experiment in *A. pusense* RIM-Id RfA adds further proof to the hypothesis that C-P lyase is the pathway used by *Agrobacterium* to degrade glyphosate. A successful knockout of *phnGHIJ* from in *A. pusense* RIM-Id RfA was created through replacement with a spectinomycin-streptomycin resistance gene using a double crossover. The knockout was confirmed by PCR.

Removing *phnGHIJ* removed the ability of *A. pusense* RIM-Id RfA to grow on glyphosate as the sole source of phosphorus. By conjugating a genomic library made from the wild type (non-knockout) version of RIM-Id, growth on glyphosate as sole source of phosphorus was restored. Both optical density, and ELISA quantification of the remaining

glyphosate confirmed this. After sequencing and annotation, the complementing cosmid was found to contain the portion of the C-P lyase *phnFGHIJKLO*, along with other miscellaneous genes within the cosmid are likely 'along for the ride' because the method used to construct the library preferentially selects large inserts (Cheng et al., 2014). The fragment of the *phn* operon within the complementing cosmid contained both the deleted portion, and additional C-P lyase genes.

The restoration of growth by the cosmid shows that we can say that *phnGHIJ* is essential for glyphosate degradation. This fits previous work in other bacterial species in which phnJ and phnI perform C-P bond breaking functions, and PhnG and PhnH are both accessory proteins of PhnI and a part of the protein complex that performs the C-P bond breaking cycle (Hove-Jensen et al., 2014; Stosiek et al., 2020).

The spectinomycin-streptomycin resistance gene used to replace *phnGHIJ* came from a plasmid containing the omega interposon, which was synthesized without the transcriptional terminator sequences (Blondelet-Rouault et al., 1997). However, it is important to note that previous work predicted *phnGHIJKLO* to be transcribed as a single transcriptional unit (Masotti et al., 2021). This is exactly the portion (plus *phnF*) that was isolated through the genomic library complementation. Though terminator sequences were not added with the knockout design, it is likely that the deletion is polar on *phnKLO*, and therefore replacing them on a plasmid is necessary. Further work is required to find the effects of the deletion on downstream genes. Replacement of *phnCDE$_1$E$_2$* was not necessary, likely due to the fact that *phnCDE$_1$E$_2$* are part of a different predicted transcriptional unit

(Masotti et al., 2021). The repressor, PhnF was also replaced in our sequence, though it is transcribed divergently from the rest of the *phn* operon. However, PhnF is a negative regulator, normally turning off expression of C-P lyase, and an inability to express PhnF is likely to result in C-P lyase overexpression rather than under expression (Hove-Jensen et al., 2014). Though it is likely that our knockout also removed PhnF expression (and our cosmid restored it), it is unlikely that this alone had a negative effect on glyphosate degradation when compared to knocking out the known C-P lyase components PhnGHIJ (Hove-Jensen et al., 2014). The deletion mutant was able to grow normally when ortho-phosphate was provided even with predicted C-P lyase over expression.

This work represents confirmation that *Agrobacterium* uses the C-P lyase system to break the carbon phosphorus bond in glyphosate and use it as a source of phosphorus.

4.4.4 Growth on, and degradation of, glyphosate

Four strains of *Agrobacterium* were found to grow on glyphosate as the sole source of phosphorus. *A. tumefaciens* GSA6-1 and GSC6-1, along with *A. pusense* RIM-Id RfA grew well on glyphosate and on our media, while *A. fabrum* C58 RfB had a significant lag phase when growing on glyphosate as the sole source of phosphorus, but eventually grew to a similar optical density as it did when provided with ortho-phosphate. Overall, *A. fabrum* did not grow as well when ortho-phosphate was provided as either *A. tumefaciens* GSA6-1 or GSC6-1, or *A. pusense* RIM-Id RfA (Appendix B Figure B.5), and this along with the long

lag phase is likely what accounted for previous erroneous understanding that C58 is not able to degrade glyphosate.

Glyphosate remaining after 96 hours of growth was quantified using and ELISA assay. Growth on glyphosate as a sole source of phosphorus did in fact result in the degradation of that glyphosate, however good growth was not perfectly matched with full glyphosate degradation. For example, *A. tumefaciens* GSC6-1 degraded only 80% of the available glyphosate after 96 hours, while *A. tumefaciens* GSA6-1 and *A. pusense* RIM-Id RfA degraded 100%, despite them all reaching a relatively similar optical density at 96 hours. *A. fabrum* C58 RfB by contrast, grew poorly but degraded all of the available glyphosate. This difference shows that glyphosate degradation is not perfectly matched to optical density, and further study with additional replicates and higher starting glyphosate concentrations is warranted. More generally, ELISA quantification using a commercial kit is an accessible method that can be used by microbiology labs to add proof of glyphosate degradation beyond simple growth/no growth assays.

4.4.5 Phn operons

The *phn* operons were pulled from the sequenced genomes and annotated to fit the *phn* naming convention. Operons were found to match the structure of a glyphosate degrading *A. tumefaciens* (Masotti et al., 2021). It also matched the gene structure of *A. radiobacter*, however previous analysis found a second *phnM* in *A. radiobacter,* 1000 kbp away from the main *phn* operon (Hove-Jensen et al., 2014). The second *phnM* has not been found in

subsequent independent analysis or in our strains (confirmed by BLAST), and it is likely that this was a slight misannotation that has been corrected in the decade since it was first reported (Masotti et al., 2021). Additionally, none of these strains contained *phnP*, and all of them contained *duf1045*. This agrees with previous work that shows that even though *phnP* has a role in carbon-phosphorus bond breaking, it is not found in phosphonate degrading *Agrobacterium* (Hove-Jensen et al., 2014; Masotti et al., 2021). PhnP breaks the unstable cyclic structure that is created by PhnJ. It may be that Duf1045 performs this function, however this is speculation because the true function of Duf1045 is still unknown.

All strains contained the full complement of C-P lyase genes expected in *Agrobacterium*, and all strains were able to degrade glyphosate. It is likely that glyphosate degradation is very widespread within *Agrobacterium*, and that a full C-P lyase operon in *Agrobacterium* is strongly indicative of the ability to degrade glyphosate.

4.5 Conclusions

Glyphosate degradation appears to be widespread in *Agrobacterium*, with two isolates of *A. tumefaciens* (GSA6-1 and GSC6-1), *A. pusense* RIM-Id RfA, and *A. fabrum* C58 RfB all able to grow on glyphosate as their sole source of phosphorus. Through de novo hybrid genome sequencing and assembly, two closely related *A. tumefaciens* genomes, an *A. pusense* genome, and another copy of the lab strain *A. fabrum* C58 are added to the body of *Agrobacterium* knowledge. These 4 strains have undergone significant genome rearrangement, specifically within and between the chromid, oncogenic, and non-oncogenic

plasmids. This adds further weight to the theory that *Agrobacterium* exhibits hyper-flexibility in its genome.

All 4 strains contain the expected complement of C-P lyase genes within the *phn* operon. In contrast to the genome rearrangements, there was remarkable homogeny within the *phn* operon, with the same gene order found in all 4 strains. Knockout and complementation experiments further showed that glyphosate degradation in *A. pusense* RIM-Id RfA relies on an intact *phn* operon. This adds further proof that *Agrobacterium* uses the C-P lyase system to break the carbon phosphorus bond in glyphosate. The library used to complement the knockout, along with a library made from the related *A. tumefaciens* GSA6-1 are now available to select for complementation of other functions in other Pseudomonadota, especially Rhizobiaceae. In future, hopefully these libraries can be used to further our understanding of the functions of *Agrobacterium*, both related to and unrelated to pesticide degradation.

Finally, glyphosate degradation has been shown to be common in *Agrobacterium*, and that glyphosate degrading *Agrobacterium* can be isolated from agricultural fields. When taken together, this may bring us closer to understanding why glyphosate rarely becomes a long-term contaminant in fields.

Chapter 5

General Conclusions and Next Steps

5.1 Conclusions

5.1.1 Media

The media and methods presented here were able to differentiate between glyphosate-tolerant phosphorus scavengers, and true glyphosate degradation. Phosphorus contamination levels were able to be kept extremely low (0.78 mg/L in liquid culture) which functions to minimize false positives, bacteria that appear to grow on glyphosate but in actuality are growing on contaminating phosphorus.

The handling procedures to ensure low levels of phosphorus contamination, along with the controls, served as a foundation to test strains already isolated within the laboratory (*Agrobacterium pusense* RIM-Id and *A. fabrum* C58). Additionally, this media was used to make enrichment cultures to compare enrichment for organisms able to use glyphosate as a sole source of phosphorus, and for organisms using ortho-phosphate and not exposed to glyphosate.

5.1.2 Enrichment

Glyphosate degrading communities were successfully enriched from soil previously exposed to glyphosate. The glyphosate degrading communities were also compared to parallel enrichments using easily accessible ortho-phosphate as the sole source of phosphorus. It was

found that Rhizobiaceae in general, and *Agrobacterium*, *Ochrobactrum* and *Ensifer* specifically, were enriched by glyphosate as the sole source of phosphorus. By contrast, *Klebsiella* and *Pseudomonas* were the primary components of the ortho-phosphate enrichment cultures, and were enriched primarily by the laboratory conditions rather than through a limited phosphorus source. Ordination plots using both Bray-Curtis and weighted UniFrac confirmed that communities separated strongly by phosphorus source (ortho-phosphate vs glyphosate) but not strongly by inoculant source (soil from a soy field or from a corn field). Weighted UniFrac, which takes into account species phylogeny, further separated the samples by phosphorus source, reflecting the evolutionary distance between the major species selected under each of the phosphorus conditions.

The ubiquity of Rhizobiaceae in agricultural soil, along with its potential plant growth promoting effects, has implications for glyphosate use in agriculture. Though adding a herbicide that is toxic to many bacterial species will undoubtedly cause shifts to soil ecology, there is the potential that some of these shifts could be benign or even beneficial.

The glyphosate degrading enrichment cultures also served as a source from which glyphosate degrading strains of bacteria were isolated. These strains were studied further and their glyphosate degradation potential, along with their genomes, were compared to related species. Most of the enrichment cultures were able to degrade all of the available glyphosate (0.025 g/L in 96 hours). There may be bioremediation applications if this culture can be optimized.

5.1.3 *Agrobacterium*

The validated phosphorus free media was used to test glyphosate degradation using previously isolated *Agrobacterium*, along with *Agrobacterium* isolated from the enrichment cultures. Three species of *Agrobacterium* were tested, *A. pusense* RIM-Id RfA, *A. fabrum* C58 RfB, and two strains of *A. tumefaciens*: GSA6-1 and GSC6-1. All isolates were sequenced using a hybrid approach using long and short reads, and then assembled, annotated, and confirmed. All strains were found to degrade glyphosate, and to contain a full C-P lyase operon.

Knockout experiments deleted the C-P lyase genes *phnGHIJ* from the *A. pusense* RIM-Id RfA. Growth tests showed that the deletion mutant was unable to grow on glyphosate as the sole source of phosphorus, but growth was normal when ortho-phosphate was provided. A genomic library was created from wild type (glyphosate degrading) *A. pusense* RIM-Id RfA Using this library, a cosmid clone was isolated that restored growth on glyphosate in the knockout strain *A. pusense* RIM-Id RfA *ΔphnGHIJ*. The complementing cosmid was found to contain a portion of the C-P lyase operon, including the interrupted genes. This knockout and complementation experiment adds additional proof that the C-P lyase system is required for glyphosate degradation in *A. pusense*, and C-P lyase operon comparison suggests that it is also responsible for glyphosate degradation in other *Agrobacterium* species.

5.2 Future work

5.2.1 Media

The developed media and set of controls should act as a base for further study of glyphosate degradation for use as a phosphorus source in bacteria. This can be done either through direct growth tests of strains of interest, or through additional enrichment cultures. If additional enrichment cultures are performed, the pH of the media, nature of the inoculant source, temperature, or carbon source should be varied and compared to the results seen in this work.

While Rhizobiaceae in general and *Agrobacterium* specifically were focused on in this work, other bacterial families contain glyphosate degrading organisms and these warrant further investigation. To name a few, glyphosate degradation has been found in *Chryseobacterium* (of the Flavobacteriales order), *Nostoc* (of the Cyanobacteria), and *Burkholderia* (of the Betaproteobacteria class) (Hove-Jensen et al., 2014; W. Zhang et al., 2022). Though this media does a good job at growing Rhizobiaceae, *Pseudomonas* and *Klebsiella* (when an alternative phosphorus source is provided), it likely needs to be optimized for other organisms by varying pH, varying carbon source, or by adding supplements.

5.2.2 Enrichment

Enrichment cultures showed enrichment for glyphosate degrading Rhizobiaceae, however only *Agrobacterium tumefaciens* was isolated on solid media. Additional work should be

devoted to isolating other glyphosate degrading species from these enrichments and testing their glyphosate degradation potential in pure culture. The C-P lyase operon of *Ochrobactrum rhizosphaerae* and *Ensifer adherens* should be sequenced and compared to *A. tumefaciens* GSA-1 and GSC-1.

The cultures were grown as mixed communities. Further testing should be performed to compare the glyphosate degrading potential of the community to the isolated major components of that community, and to re-constituted synthetic communities. Though carbon-phosphorus cleavage is likely a self-contained process, there may be synergistic effects allowing for greater glyphosate degradation when grown as a mixed culture rather than as pure cultures.

Because Rhizobiaceae were enriched by the glyphosate degrading enrichment cultures, and because glyphosate degradation appears widespread in Rhizobiaceae, additional Rhizobiaceae strains should be tested to see if glyphosate degradation is ubiquitous. If Rhizobiaceae strains are found that do not degrade glyphosate, the genomic libraries created and tested in Chapter 4 should be used to select complementing cosmids to help uncover additional genes and processes necessary for glyphosate degradation. Plant growth promoting Rhizobiaceae should also be screened for their interactions with glyphosate. We can safely expect plant growth promoting bacteria (wild or applied) to interact with glyphosate in agricultural systems, especially agricultural systems that are using glyphosate in place of tillage out of concern for soil health. Is it possible that glyphosate application may shift the microbiome towards one that is beneficial to crop production?

5.2.3 *Agrobacterium*

Three species of *Agrobacterium* were tested in this study, and all were found to degrade glyphosate. However, the *Agrobacterium* genus is diverse, and glyphosate degradation may not be ubiquitous. Like Rhizobiaceae in general, additional *Agrobacterium* species should be selected and tested for glyphosate degradation. Ideally, the strains chosen should be widely spaced over the entire *Agrobacterium* species tree.

This study has added additional proof that C-P lyase generally and *phnGHIJ* specifically is a necessary component for glyphosate degradation by *Agrobacterium*. However, *duf1045* appears to be common in the C-P lyase operon of *Agrobacterium* and yet its role in carbon-phosphorus bond breaking, if any, remains unknown. Additional work should be done to create *duf1045* knockouts and replacements, followed by screening for glyphosate degradation.

The *Agrobacterium* genus appears to be hyper-diverse and with frequently rearranged genomes (Weisberg et al., 2023). This study adds additional genome sequences of *A. pusense* RIM-Id RfA, *A. tumefaciens* GSA6-1 and GSC6-1, and *A. fabrum* C58 RfB. To add to the understanding of *Agrobacterium* genome diversity, these genomes will be archived and made available to the public through an NCBI BioProject, accession PRJNA1198028.

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Appendix A

Supplemental Media Recipe

Table A-5-1 Autoclaved base media

NaCl	10 g
$MgSO_4+7H_2O$	0.5 g
$CaCl_2$	0.015 g (easier to add as 1mL of 15g/L stock)
KCl	1.6 g
NH_4Cl	1.0 g
Milli-Q Water	To 900 mL

The base media is made and diluted to 900 mL with ultrapure milli-Q water before autoclaving. If a solid agar media is desired, all dry chemicals above are instead added to the filter sterilized portion (table A-2), and 15 g of Noble agar is instead autoclaved in 900 mL milli-Q water. Noble agar solidifies more consistently if it is autoclaved without added salts.

Table A-5-2 Filter Sterilized Portion

HEPES-Acid	11.9 g (0.5mol)
HEPES-Sodium Salt (Base)	13.0 g (0.5mol)
Trace Elements* (see below)	1 mL
D-Glucose	3 g
Milli-Q	100 mL
Phosphorus source (e.g. glyphosate, NaH_2PO_4)	Use the same molarity of phosphorus

HEPES buffer components must be dissolved before glyphosate is added, as glyphosate does not dissolve well in unbuffered media. This portion of the media is heat sensitive, and is therefore filter sterilized and added to the base media from table A-1 after the base media is autoclaved. When filter sterilizing, dedicated or single use syringes are used to avoid phosphorus cross-contamination.

Table A-3 Trace Elements Solution

NaEDTA	1 g
K_2SO_4	1 g
H_3BO_3	0.1 g
$ZnSO_4*7H_2O$	0.1 g
$CuSO_4*7H_2O$	0.05 g
$MnCl_2*4H_2O$	0.05 g
$Na_2MoO_4*2H_2O$	0.1 g
$CoSO_4$	0.1 g
Milli-Q	1 L

The trace elements are used sparingly and in small amounts such that dedicated stocks of these chemicals are not required, unlike the main media components. The trace elements are stored at 4°C after autoclaving.

Appendix B

Supplemental *Agrobacterium* Growth Curves

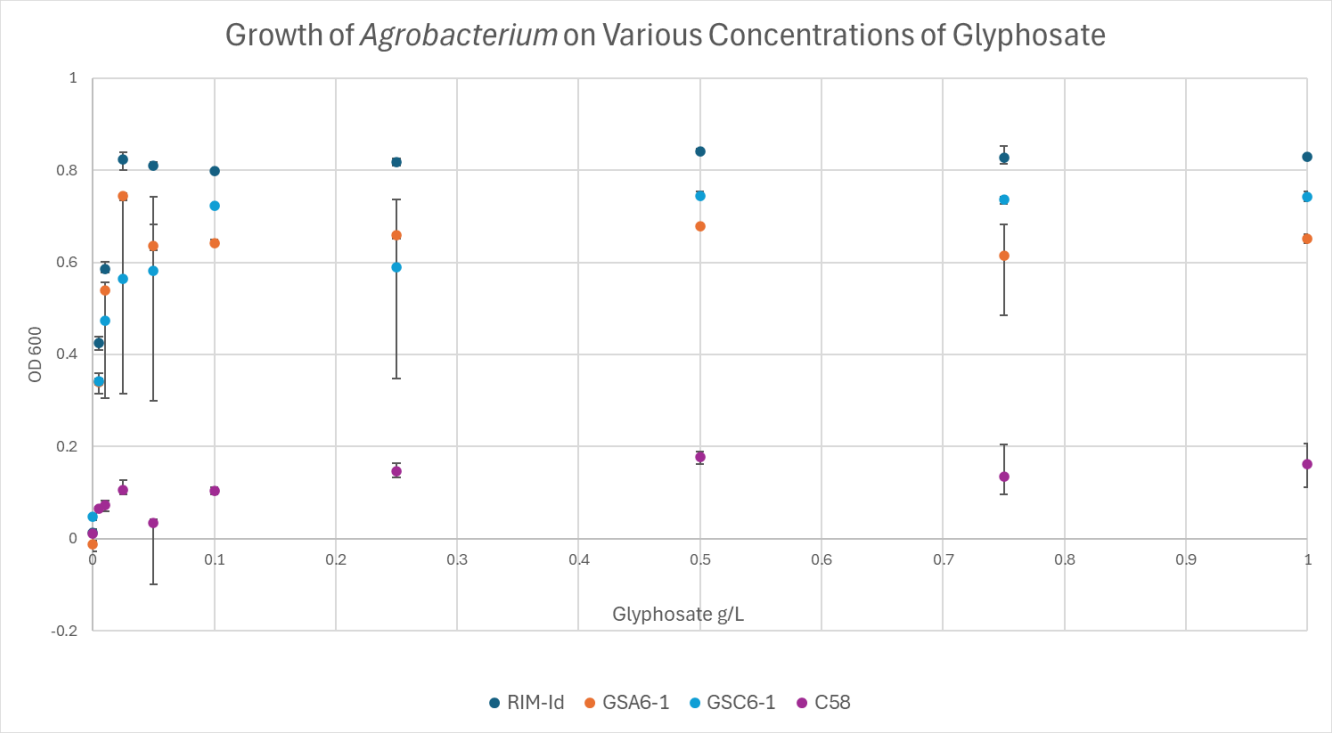


Figure B.1 Growth of *Agrobacterium* On Various Levels of Glyphosate: To find the point at which glyphosate becomes limiting to growth, glyphosate degrading *Agrobacterium* were grown on various concentrations of glyphosate. Shown is the growth at 96 hours (at or beyond the maximum growth) plotted by the concentration of glyphosate in grams per liter. Error bars show the highest and lowest measurement of the triplicate.

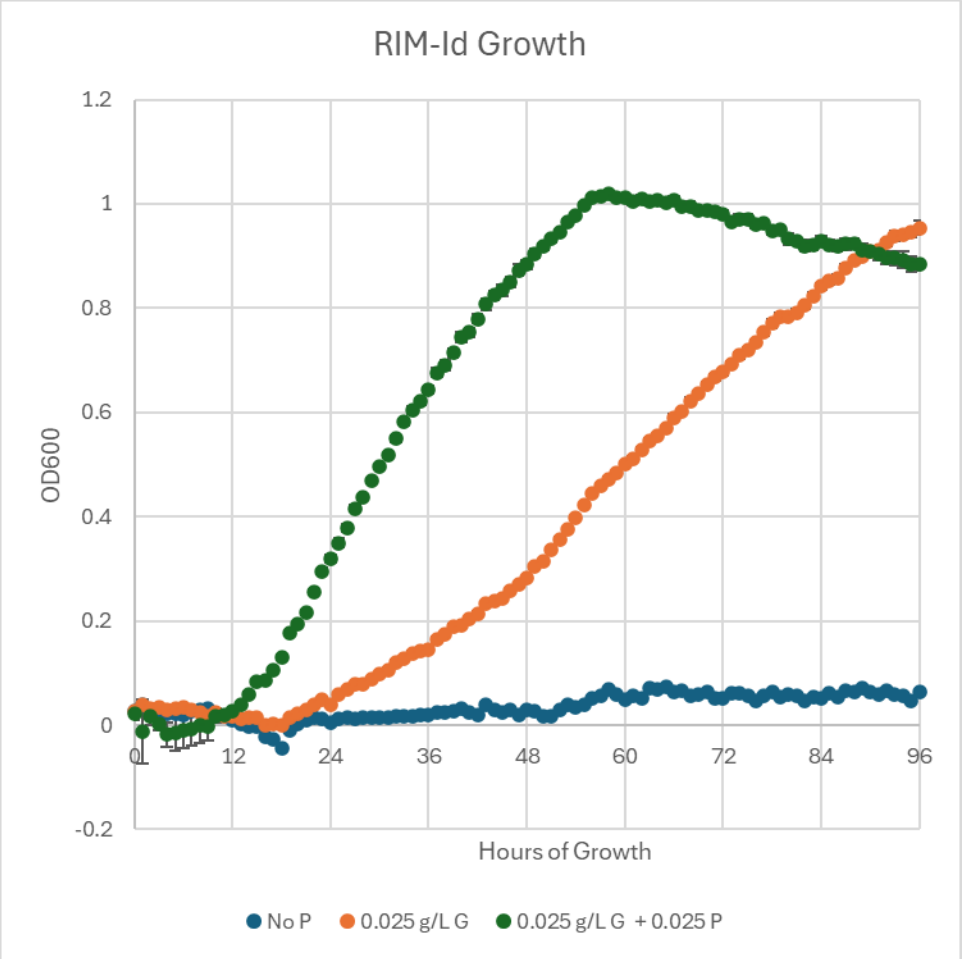


Figure B.2 Growth of *Agrobacterium pusense* RIM-Id RfA: *A. pusense* RIM-Id grows well both on glyphosate as sole source of phosphorus and when ortho-phosphate is provided. Growth is not seen when no phosphorus source is added.

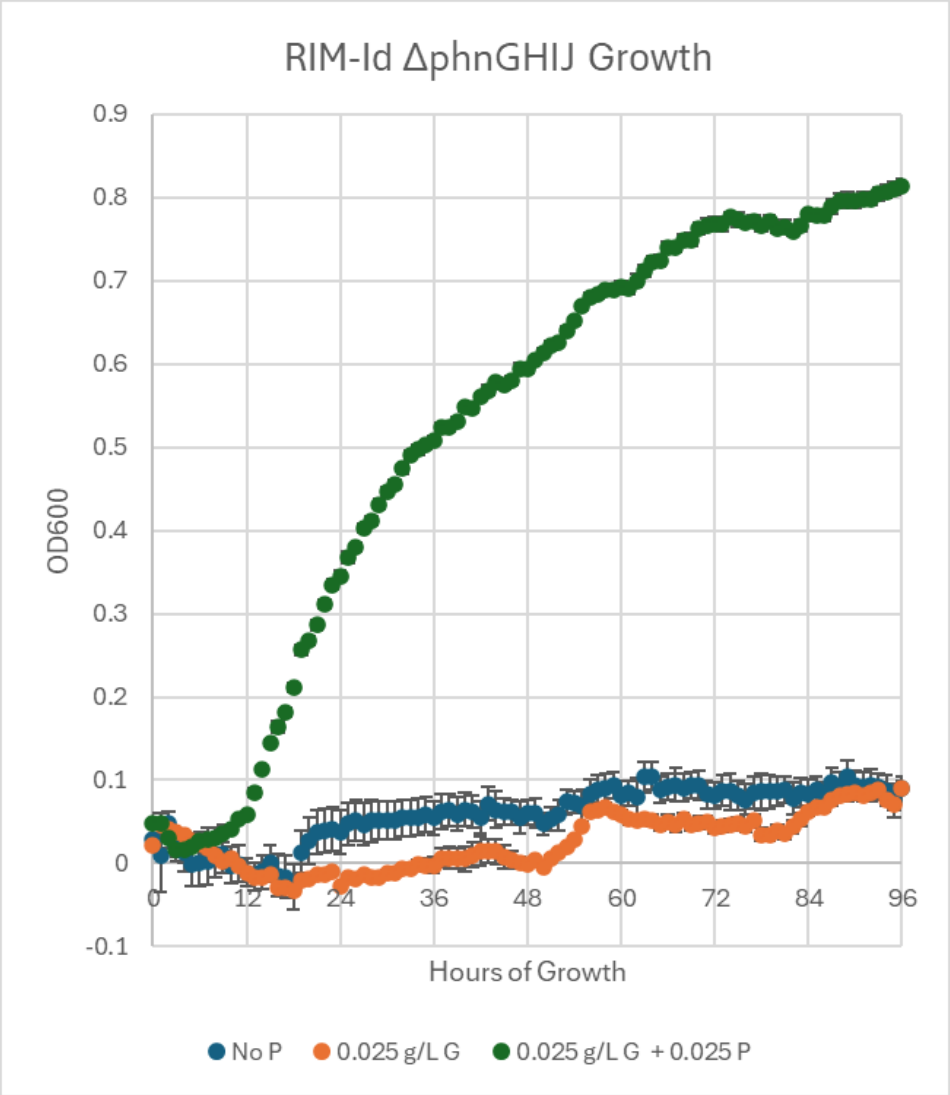


Figure B.3 Growth of *Agrobacterium pusense* RIM-Id RfA: *A. pusense* RIM-Id ΔphnGHIJ grows well when ortho-phosphate is provided. Growth is not seen when glyphosate is the sole source of phosphorus or when no phosphorus source is added.

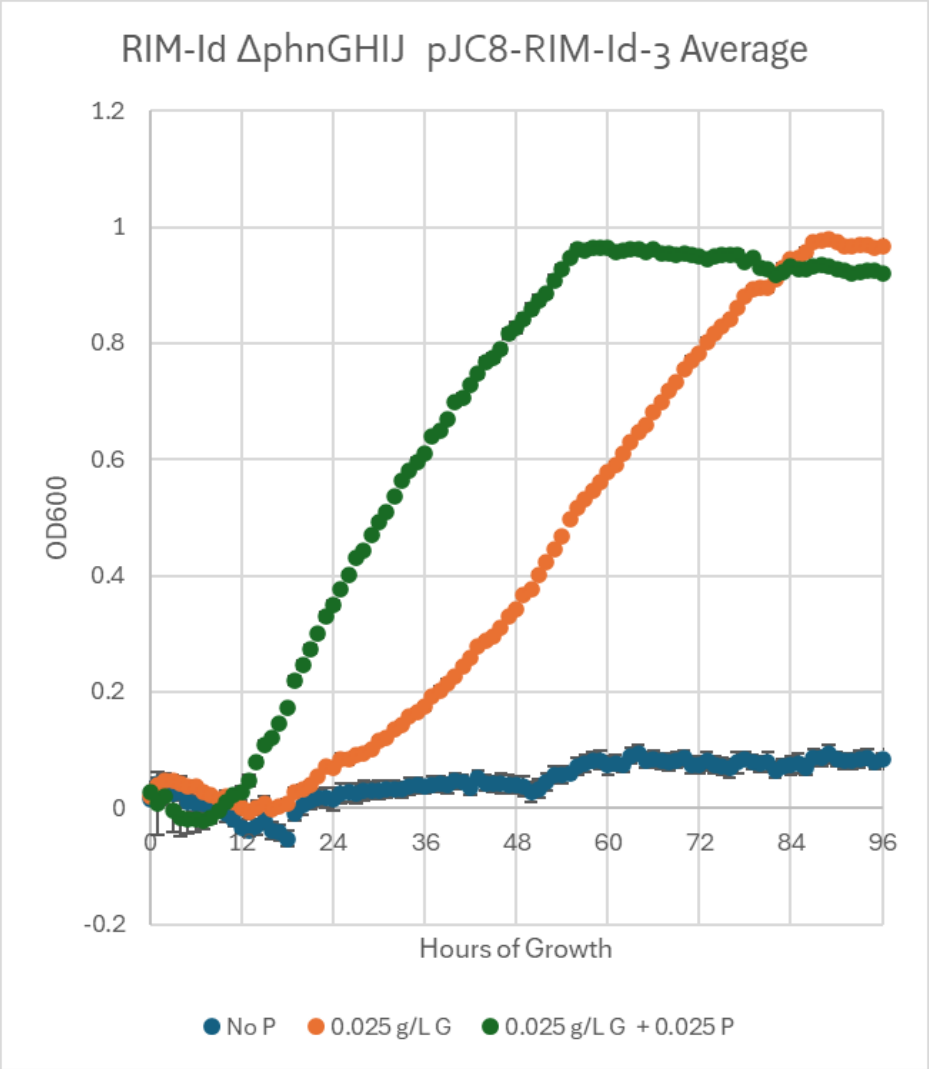


Figure B.4 Growth of *Agrobacterium pusense* RIM-Id RfA ΔphnGHIJ pJC8-RIM-Id-3: *A. pusense* RIM-Id ΔphnGHIJ with the pJC8-RIM-Id-3 cosmid grows well both on glyphosate as sole source of phosphorus and when ortho-phosphate is provided. Growth is not seen when no phosphorus source is added.

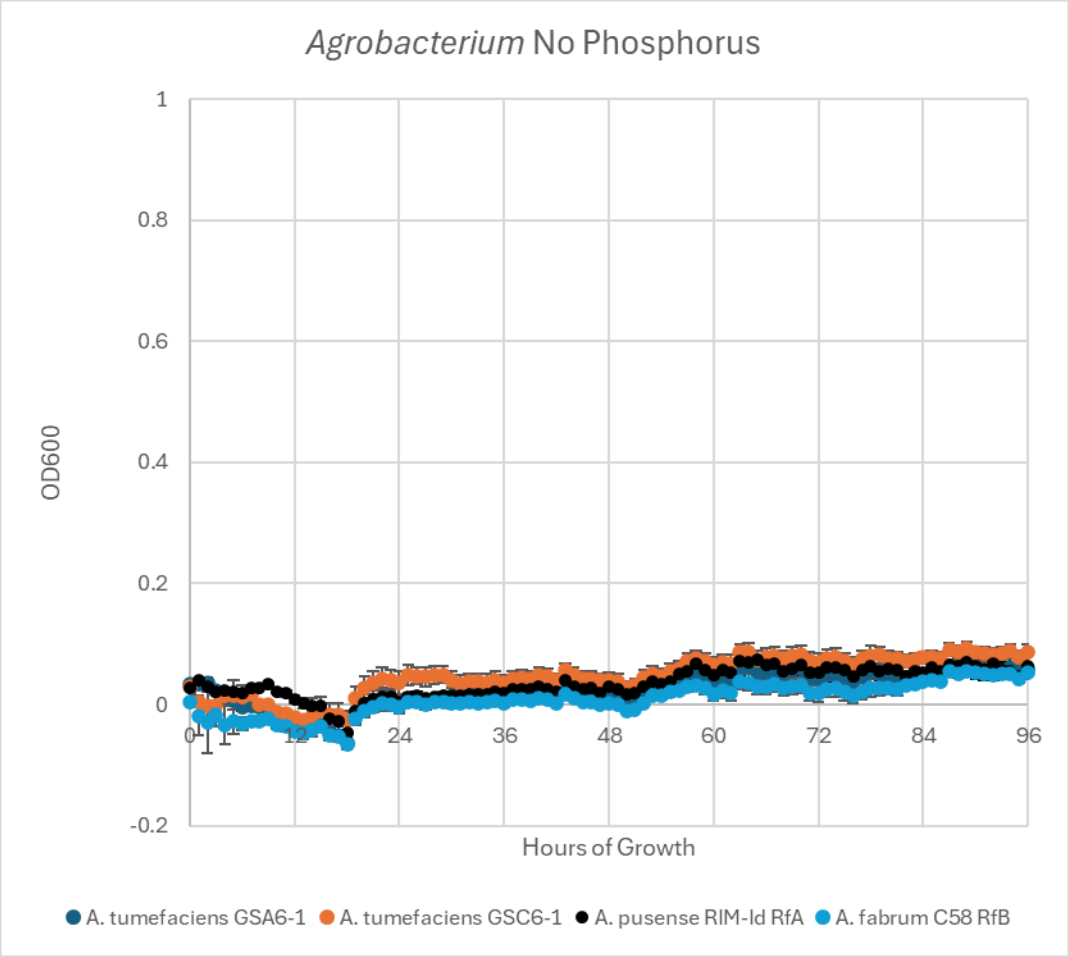


Figure B.5 Growth of *Agrobacterium* Without Added Phosphorus:Growth of strains when no phosphorus is provided. Optical density has been corrected by subtracting the optical density of the phosphorus free sterile control. In all cases the media is sufficiently free of phosphorus to preclude growth.

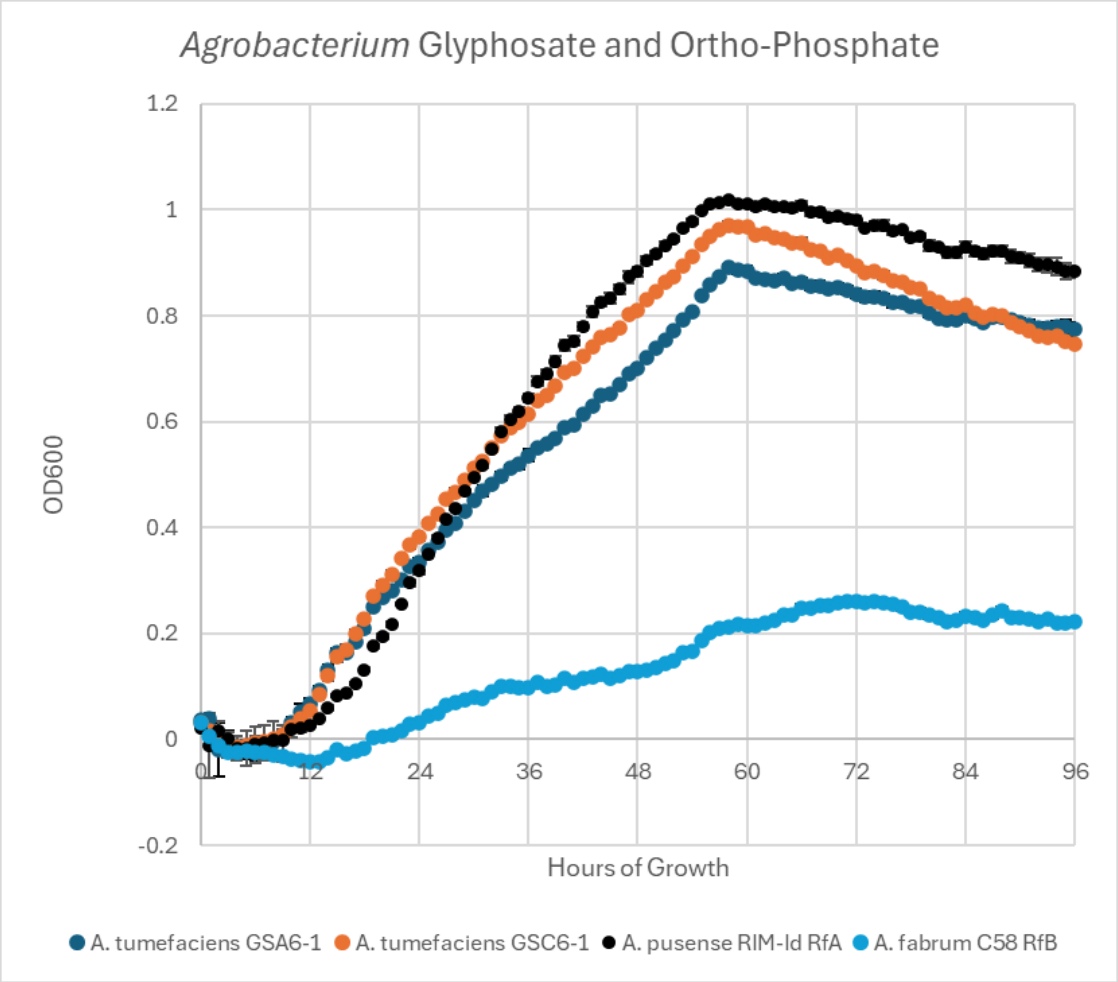


Figure B.6 Growth of *Agrobacterium* With Glyphosate and Ortho-Phosphate:Growth of strains when both glyphosate and ortho-phosphate are provided. Optical density has been corrected by subtracting the optical density of the phosphorus free sterile control. In all cases the media allows for growth if ortho-phosphate is provided.