CONSTRUCTION OF GREEN FLUORESCENT PROTEIN PLASMIDS FOR LABELING OF HERBICIDE-DEGRADING *DELFTIA ACIDOVORANS* MC1071

BY

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THESIS

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ABSTRACT

Each year, approximately 46 million pounds of 2,4-dichlorophenoxyacetate (2,4-D) (EPA, 2005) and 4 million pounds of 2,4-dichlorophenoxypropionate (2,4-DP) (EPA, 2007) herbicides are applied for commercial or residential usage in the US. Accidental runoff of herbicides such as 2,4-D and 2,4-DP from human applications is toxic to non-target organisms and harmful to the environment. The main mode of removal for most herbicides in nature is biodegradation. In natural environments, herbicides and substrates are non-uniformly distributed, and microorganisms compete to degrade these compounds. The favorability of one microbe over another depends on many factors, including the herbicide and substrate concentrations.

The objective of my research is to construct two green-fluorescent protein (GFP) plasmids to tag a specific strain of *D. acidovorans* (MC1071) for visual distinction from one or more competing microbes in a microfluidic pore structure that contains spatially varying concentrations of 2,4-DP, 2,4-D, and/or oxygen. Two GFP plasmids, designated pKN*lacgfp* and pKN*tfdgfp*, were constructed and successfully transformed into MC1071. One resultant strain, MC1071/pKN*lacgfp*, constitutively expressed GFP, while the strain MC1071/pKN*tfdgfp* expressed GFP only when herbicide degradation was induced. The MC1071/pKN*lacgfp* strain will now be visually distinct from other *D. acidovorans* strains when grown in micromodels, allowing for monitoring of competition between MC1071 and other strains. The MC1071/pKN*tfdgfp* strain will allow distinction of active and inactive MC1071 biomass in micromodels, thus facilitating the study of bacteria adaptation in micromodels.

Thank You to My Family: all the Ng's and Yim's. And a Special Dedication to the Newest Member of the Family Abigail Joy Ng.

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CHAPTER 1: INTRODUCTION

Human use of herbicides comes with the possibility of introducing the herbicides' toxicity into nature. Many herbicides are persistent, mobile, and toxic to non-target organisms, making accidental or uncontained herbicide runoff a major risk to the environment. For example, the herbicide 2,4-dichlorophenoxyacetate (2,4-D), which accounts for approximately 10% of the total annual herbicide usage in the US, can be found in approximately 660 agricultural or home-use herbicide products in the United States (EPA, 2005). 2,4-D has a degradation half-life of 6.2 days in aerobic soil conditions, and 41 to 333 days in anaerobic soil conditions (EPA,2005). It is an intermediate to very mobile chemical based on soil thin layer chromatography studies, and can pose a threat to non-target organisms long after and far from the point of application (EPA, 2005). Another herbicide similar to 2,4-D is 2,4-dichlorophenoxypropionate (2,4-DP). 2,4-DP has a half-life of 14 days in aerobic aqueous conditions, and 159 days in anaerobic aqueous conditions (EPA, 2007). It is also a mobile chemical and can pose the same threat to non-target organisms as 2,4-D.

The main mode of removal for most herbicides is biodegradation. Degradation of 2,4-D and 2,4-DP by the bacterial strain *Delftia acidovorans* MC1 in batch cultures and by enzymes RdpA and SdpA isolated from MC1 have been studied (Müller, 1999, 2001; Westendorf 2006). These studies have given much understanding to the biochemical activity and kinetics of 2,4-D and 2,4-DP degradation. However, biodegration processes become more complex when physical phenomena such as mass transport by diffusion or advection become significant; these processes come into effect in heterogeneous settings such as soil matrices. We can study these physical phenomena with the help of micromodels – small pore systems etched into silicon wafers which simulate soil systems.

Many previous studies have been performed with the inoculation of bacterial cultures into micromodels. Different aspects of biomass growth have been studied in micromodels, including relationships between hydraulic flow and pore patterns, and patterns of biomass accumulation (Paulsen *et al.*, 1997; Dupin and McCarty, 1999; Zhang *et al.*, 2010), the relationship between substrate availability and biomass migration, density and permeability (Kim and Fogler, 2000; Nambi *et al.*, 2003), and the relationship between transverse nutrient mixing and bioactivity (Thullner *et al.*, 2001).

These past studies laid the groundwork for more complex studies of bioactivity in micromodels, where biomass growth and hydraulic flow patterns interchangeably affected one another. For example, a study of microbial adaptation in micromodels has been performed (Yoon *et al.*, submitted 2011). In this study, a derivative of bacterial strain MC1 which can only grow on the substrate (R)-2,4-DP, MC1071, was grown in micromodels. MC1071 had previously been shown to adapt to utilizing 2,4-D as a substrate in batch cultures (Leibeling *et al.*, submitted 2011). In the micromodel experiment, MC1071 was first allowed to establish stable growth with (R)-2,4-DP. Subsequently, the bacteria in the micromodel were fed a mixture of 2,4-D and (R)-2,4-DP to encourage adaption to the new substrate. New biomass was observed as 2,4-D was fed. The new biomass growth likely affected patterns of substrate mass transfer, as well as bioactivity in the micromodel. For example, previously active biomass in some pores may have become inactive as more biomass consumed oxygen and the herbicides. Unfortunately no tools were available to distinguish between biomass that was still active for substrate degradation from biomass that may have become inactive. The study was therefore limited in terms of understanding the changes in transient availability of substrates to microorganisms after alteration in biomass patterns. Although only strain MC1071 was evaluated in the micromodel,

the presence of other microbes would also affect substrate mass transfer and bioactivity. For example, when two different bacterial strains grow together, they may compete for nutrients. This competition can cause selective pressures against the strains to find more efficient ways of surviving, either by adaptation or by migrating to zones that are more advantageous to growth. To perform these competition studies, methods must be established which will distinguish between MC1071 and other bacterial strains.

The goal of my thesis work was to develop new tools which will allow for visual distinction of specific *D. acidovorans* strains. My specific objectives were to label MC1071 with a constitutively expressed green-fluorescent protein (GFP) for visual distinction from other bacterial strains, and to label MC1071 with a green fluorescent protein that is induced by biodegradation of the herbicide 2,4-DP or 2,4-D. To isolate labelled-MC1071 strains, the introduction of the GFP gene was coupled with resistance to the antibiotic kanamycin. This was accomplished by the construction of two GFP plasmids with kanamycin resistance. The two GFP plasmids were different in that one of the plasmids was a derivative of the other in which the expression of GFP only occurs when herbicide degradation is active. With the successful GFP-labeling of MC1071 strains, these strains can be employed in micromodels for a more in-depth understanding of the relationships between substrate bioavailability and bioactivity.

CHAPTER 2: BACKGROUND

The herbicides 2,4-dichlorophenoxyacetic acid (2,4-D) and (R,S)-2,4-dichlophenoxypropanoic acid ((R,S)-2,4-DP), and various bacterial strains of *Delftia acidovorans* that degrade them, form an ideal system of substrates and degrading bacteria suitable for studying microbial adaptation and competition phenomena. These studies will be carried out in micromodels, which are small pore systems etched into silicon wafers to simulate soil systems. To facilitate observation of different *D. acidovorans* strains in the micromodels, *D. acidovorans* strain MC1071 will be labeled with green-fluorescent protein (GFP). The expression of GFP from these plasmids will be controlled by two different promoters: a *lac* promoter that allows for constitutive GFP expression in *D. acidovorans*, and a *tfdC* promoter that allows for inducible GFP expression in *D. acidovorans*.

2.1. DICHLOROPHENOXYALKANOIC ACID HERBICIDES

The two herbicides of interest in our study are 2,4-D (Figure 1) and 2,4-DP (Figure 2). Both herbicides are of the phenoxyalkanoic acid family of herbicides used in post-emergence control of broadleaf weeds. These herbicides cause an increase in the target plants' cell-wall plasticity, biosynthesis of proteins, and production of ethylene, thus leading to uncontrolled cell division, growth, and vascular tissue damage (EPA, 2005 and 2007). Estimated annual usage of 2,4-D was 46 million pounds between 1993 to 1999 (EPA, 2005), and of 2,4-DP was 4 million pounds in 2007 (EPA, 2007).

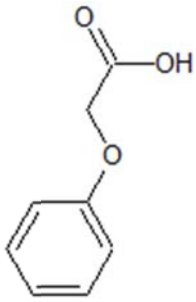

Figure 1: Chemical Structure of 2,4-dichlorophenoxyacetic acid (2,4-D)

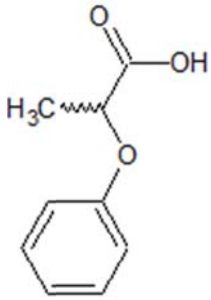

Figure 2: Chemical Structure of 2,4-dichlophenoxypropanoic acid (2,4-DP)

The US EPA has set 2,4-D's maximum contamination levels at 70ppb. Although residential and commercial exposures of these herbicides to humans do not reach levels of concerns set by the EPA (EPA, 2005 and 2007), the herbicides are considered as part of an aggregate chemical exposure to humans through food and drinking water (EPA, 2005 and 2007). The herbicides are also of concern due to their low soil-binding affinity and mobility (EPA, 2005 and 2007), which cause exposure to non-target plants and animals above levels of concern (EPA, 2005 and 2007).

The structure of 2,4-DP, as shown in Figure 2, contains one chiral carbon center, making it an optically active molecule. Only the R-enantiomer of 2,4-DP, (R)-2,4-DP, is active as a herbicide. 2,4-DP's racemic form was first registered for use as an herbicide in the 1960s. In the 1980s, technologies were developed to produce 93-95% R-enantiomer enriched forms of the

herbicide, known as 2,4-DP-p. Between 1996 and 2007, the EPA requested and received from all manufacturers of 2,4-DP voluntary conversions of their product formulation to the enriched herbicide 2,4-DP-p. (EPA, 2007).

2.2. 2,4-D AND 2,4-DP DEGRADING BACTERIA

A wide range of bacterial strains have been reported to degrade 2,4-D including, but not limited to, *Acinetobacter sp.*, *Alcaligenes eutrophus*, *Pseudomonas putida*, *Pseudomonas cepaci*, and *Rhodococcus erythropolis* (Häggblom, 1992). The plasmid pJP4 is responsible for 2,4-D degradation in the bacterial strains *A. eutrophus* and *P. putida* (Liu and Chapman, 1984). Hydrolase enzymes purified from *A. eutrophus* and *P. putida* also showed degradation activity towards 2,4-dichlorophenol and 4-chloro-2-methylphenol (Liu and Chapman, 1984).

Pure bacterial strains which degrade 2,4-DP are less common; these include but may not be limited to strain MH (Horvath *et al.*, 1990), later identified as *Sphingomonas herbicidovorans* (Zipper *et al.*, 1996), *Rhodoferax sp.* P230 (Ehrig *et al.*, 1997), *Ralstonia sp.* CS2 (Smejkal *et al.*, 2001), and *Comamonas acidovorans* MC1, later reclassified as *D. acidovorans* MC1 (Müller *et al.*, 1999). *D. acidovorans* is a gram-negative prototrophic bacterium, and strain MC1 was isolated from the vicinity of herbicide-contaminated building rubble (Müller *et al*. 1999). MC1 can degrade 4-chloro-2-methyl-phenoxyacetate (MCPA), 2,4-D, and both enantiomers of 2,4-DP (Müller *et al*. 1999).

In general, chlorophenol compounds are degraded by first being oxidized to chlorocatechols by a phenol hydroxylase, and then cleaved by intradiol ring-cleavage. Eventually the degradation product will enter the tricarboxylic acid cycle for complete mineralization. This degradation pathway is shown in Figure 3.

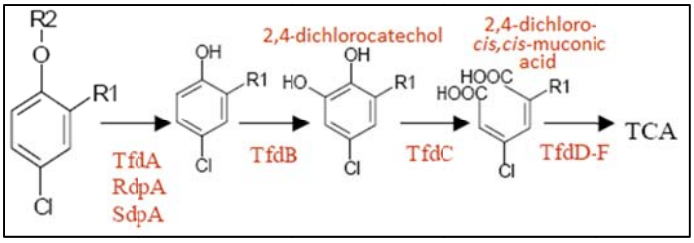

Figure 3: Degradation Pathway of Chlorophenols

In many 2,4-D degraders, the first step in the degradation pathway involves cleavage of the ester bond to yield acetic acid and 2,4-dichlorophenol. The *tfdA* gene encodes the 2,4-D/α-ketoglutarate-dioxygenase enzyme, which catalyzes the ester bond cleavage (Hofmann *et al*. 2003). This enzyme, isolated from *Ralstonia eutropha* JMP134, was shown to have activity towards 2,4-D and (S)-2,4-DP, but not (R)-2,4-DP (Saari, 1999).

Following transformation to dichlorophenol, the *tfdB-F* genes encode enzymes responsible for the complete degradation of the herbicides. The *tfdB-F* genes have controlled expression which is only active when herbicide is present. *tfdC* encodes for chlorocatechol-1,2-dioxygenase (Kõiv *et al.* 1996; Pérez-Pantoja *et al*., 2000), which catalyzes the transformation of 2,4-dichlorocatechols into 2,4-dichloro-cis,cis-muconate. We utilize the *tfdC* promoter in our studies as a genetic control for the expression of GFP. The *tfdC* promoter has basal levels of expression (Kõiv *et al.*, 1996) but is mostly activated by a TfdR regulatory protein, which is produced by a divergent *tfdR* gene in the presence of 2,4-dichloro-*cis,cis*-muconate (Vedler *et al.*, 2000). In other words, we will control GFP to only express when herbicide is degraded to the stage of 2,4-*cis,cis*-muconic acid if we control GFP expression with the *tfdC* promoter.

In MC1, two α-ketoglutarate-dependent dioxygenases are responsible for the initial ester-bond cleavage of dichlorophenoxyacetates into dichlorophenols. $RdpA_{MC1}$ and $SdpA_{MC1}$ are

stereospecific enzymes responsible for the degradation of (R)-2,4-DP and (S)-2,4-DP, respectively. $SdpA_{MC1}$ was also shown to have activity against 2,4-D and MCPA (Westendorf et at. 2003). Similar to degradation of 2,4-D by TfdA, degradation of 2,4-DP, 2,4-D, or MCPA are completed after RdpA and SdpA by the products of the *tfdB-F* genes.

rdpA genes similar to $rdpA_{MC1}$ have also been identified in *Rhodoferax* sp. P230 and *Sphingobium herbicidovorans* MH. RdpA isolated from all these strains showed similar substrate preference in the order (R)-2,4-DP, (R)-2-(4-chloro-2-methylphenoxy)propionate (MCPP), 2,4-D, and MCPA, yet different kinetics towards these substrates; namely $RdpA_{P230}$ showed slower kinetics towards similar substrates (Westendorf, 2005). *sdpA* gene sequences from P230 and MH were significantly different from $sdpA_{MC1}$ (Schleinitz *et al*. 2004; Müller *et al*. 2004). Similarities in *rdpA* but differences in *sdpA* indicate common origins to these enzymes, with the heterogeneity of *sdpA* being evidence of more recent evolution.

The herbicide-degrading strain of concern in my study was *D. acidovorans* MC1071. MC1071 is a derivative strain of MC1 that only possesses the *rdpA* gene, and therefore only degrades (R)-2,4-DP (Müller et al. 2005). In other words, since MC1071 is $SdpA^-$, it is unable to degrade (S)-2,4-DP and 2,4-D (Müller, 2005).

2.3. GREEN FLUORESCENT PROTEIN LABELING

The green fluorescent protein (GFP) was first purified from jellyfish *Aequorea* in the 1960s. (Shimomura *et al.*, 1962). The original wild-type GFP purified from *Aequorea victoria* is a protein of 238 amino acids (Prasher *et al.*, 1992) which absorbs blue light at 395 nm and fluoresces green light at 509 nm (Morin and Hastings, 1971; Ward *et al.*, 1980). Since GFP required no additional gene products from *A. victoria* to fluoresce, beginning in the 1990s GFP

became employed as a marker of gene expression in other prokaryotic and eukaryotic cells (Chalfie *et al.*, 1994). GFP quickly became an established marker of gene expression in cells and microorganisms, with many engineered derivatives developed for different applications in biology and physiology (Tsien, 1998).

One engineering of the *gfp* gene involved the insertion of random amino acids into GFP to produce a mutant GFP more suitable to monitoring under standard fluorescein isothiocyanate (FITC) excitation/emission fluorescent microscope filters; the result of this was *gfpmut3a* (Cormack *et al.*, 1996). FITC filters operate with excitation and emission windows close to 485 nm and 538 nm. As a result of mutation, *gfpmut3a* has excitation/emission wavelengths of 501/511 nm instead of 395/509 nm as in wild-type GFP (Cormack *et al.*, 1996). *gfpmut3a* also exhibits a 21-fold increased fluorescence intensity over wild-type GFP when excited at 488 nm (Cormack *et al.*, 1996).

2.4. GFP-LABELED *D. ACIDOVORANS* MC1071 IN BACTERIAL ADAPTATION AND COMPETITION STUDIES IN MICROMODELS

Given the right conditions, bacterial strains can adapt to new substrates they are exposed to; an example is MC1071 adapting to degrade 2,4-D by post-translational modification of the RdpA enzyme (Leibeling, submitted 2011). Bacterial adaptation is often studied in batch cultures in the laboratory; this approach was used to study MC1071's adaptation to 2,4-D (Leibeling, submitted 2011). Batch growth allows for conditions where bacteria cells have constant exposure to substrates in the solution until substrate exhaustion. In nature, however, bacterial growth behavior may be affected by physical heterogeneities such as substrate availability due to mass transfer limitations. Spatial variation in substrate availability may provide different selective

pressure to bacteria cells of the same strain growing in different locations, and thus lead to different rates of adaptation in different areas inhabited by the same strain.

Laboratory batch cultures are also commonly grown as pure cultures. However, cultures in nature are rarely pure, and bacterial strains must grow in proximity to one another and compete for nutrients. The competition between different bacterial strains adds pressure to each strain as less substrate will be available. Different bacteria cultures may modify themselves to more efficiently survive or out-compete other strains; they may change their morphology or location to become better recipients of nutrients, or they may adapt to using more varied kinds of substrates to sustain growth.

To study bacteria adaptation and competition under the influence of mass transfer effects in the laboratory, we can grow and monitor bacteria cultures in micromodels – small pore structures etched onto silicon wafers. Micromodels used in our studies, shown in Figure 4, measure 1 cm × 2 cm and have uniform pore structures throughout. Three inlets are available for injection of different chemicals into our micromodel. Inlets A and B are intended for the injection of different substrates, while Inlet C serves as a sodium azide inlet which terminates microbial growth downstream from the micromodel pore structure.

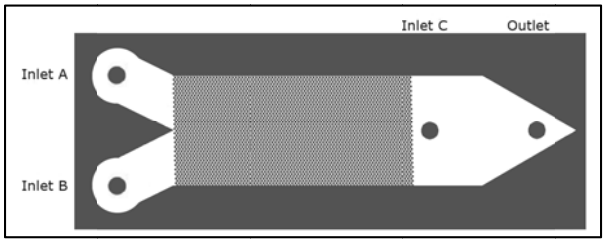

Figure 4: Schematic of Micromodel Assembly

We will inoculate the micromodels of our study with either pure cultures of MC1071, or mixed cultures of MC1071 and other herbicide-degrading strains. We intend to observe the adaptation of pure MC1071 to degrade 2,4-D by first establishing stable growth of MC1071 in micromodels on their natural substrate of (R)-2,4-DP, then switching the inlet feeds of the micromodel to an (R)-2,4-DP/2,4-D mixture. The MC1071 strain employed in these studies will be labeled with GFP. The MC1071 strain employed in these studies should only express GFP when actively degrading herbicide, so that a distinction can be made between bacterial cells that are still actively degrading the herbicide after changes in inlet substrate composition, to bacterial cells that stop being active after the change. We also wish to observe the competition of MC1071 with other herbicide-degrading strains such as MC1. We will monitor if either MC1071 or any competing strains will modify their morphology or location for the purpose of gaining competitive advantages over other cultures. For these studies, we can visually distinguish MC1071 cultures from cultures of other strains if MC1071 contains constitutively expressed GFP. For the purpose of employing GFP-labeled MC1071 in adaptation and competition micromodel experiments, an induced-GFP strain and a constitutive-GFP strain of MC1071 will be produced.

CHAPTER 3: LITERATURE REVIEW

In this study, I am developing tools to study how physical limitations of substrate mass transfer in soil systems and biomass activity affect each other. There are many existing studies of biomass activity and mass transfer interactions in micromodels whose development and experience can be drawn upon to aid in my study. I intend to construct fluorescently labeled bacterial strains that can be visually distinguished in micromodels, and thus an understanding of the fluorescent marker that I will be employing, the green fluorescent protein (GFP), and its effects on prokaryotic cells will also be beneficial.

3.1. MODELLING SUBSTRATE-LIMITED BIOMASS ACTIVITY IN POROUS MEDIA

Methods of studying biomass in micromodels were developed by Paulsen *et al.*, (1997), when a mixed bacterial culture was inoculated and grown in a 2-D porous media replica of a sandstone reservoir and observed through a microscope. The study included the development of techniques to record in-pore flow velocities through the observation of cell detachment velocities. It also classified different kinds of biofilm morphologies that may be expected in micromodel studies, such as biowebs and biofilms, and gave understanding to subsequent micromodel studies of how these different biofilm morphologies may affect convective mass transport and convective flow.

After Paulsen *et al.*'s study of bioactivity in micromodels, Kim and Fogler (2000) went on to study the different stages of growth and death that biomass can go through in the micromodel. *Leuconostoc mesenteroides* was grown in a porous micromodel in both nutrient-rich and carbon-source depleted conditions, and four stages of biomass growth and permeability were observed. Initially, permeability decreased due to biomass accumulation in the nutrient-rich phase. Next,

biomass remained constant and permeability reached a minimum with nutrient depletion. Third, biomass began to reduce due to biofilm sloughing caused by shear stress, causing an increase in permeability. Finally, biomass and permeability reached a steady state as shear stress remained below critical shear stress for sloughing. Kim and Fogler's study will give context to the visual observations of biomass in future micromodel experiments; namely it will give reference to the health or stage of biomass growth we will observe in our micromodels.

Further studies of biomass growth patterns in micromodels were performed by Dupin and McCarty (1999). In their study, a 2-D random width network pore model was used as a representation of fine sand and they grew a mixed microbial community in media with 0.34 mM acetate under aerobic conditions at a fixed flow rate. The resultant biomass showed variable growth density, with the most dense biomass growing in the up-gradient and lateral directions of substrate, and least dense in the down-gradient direction. Particles used as trackers in the pore model suggested that biomass growth caused rerouting of flow, and thus showed that biomass growth in porous structures can affect mass transfer and that such phenomena can be studied in micromodels.

With the understanding that biomass growth can affect mass transfer in micromodels, Thullner *et al.* (2001) went on to study how the affected mass transfer can then reciprocate and affect biomass growth. In their study, bacteria were grown in a 56 × 44 × 1 cm3 flow cell filled with glass beads and operated under a continuous flow of nitrate as an electron acceptor. Glucose was injected through a port to simulate a point source contamination. After 31 days, the highest amount of biomass was found near the glucose injection port where nutrient abundance was highest. It was concluded that bioactivity only occurred in zones of effective electron donor and acceptor mixing, suggesting that bioactivity was limited by transverse mixing.

Nambi *et al.* (2003) studied the same effects of mass transfer on biomass, but with a different model design and pattern of substrate feed than Thullner *et al* (2001). In Nambi *et al.*'s study, *Sulfurospirillum multivorans* was grown in a 2-D micromodel to determine how mass transfer limitations transverse to advection affected biomass growth. Nambi *et al.*'s micromodel design is much like the one that Zhang *et al.* (2010) would later use, which is a study of *D. acidovorans* cultures in micromodels which our long term project experiments are based on.

In Nambi *et al.* (2003), tetrachloroethene (PCE) and lactate were introduced from two separate inlets of the micromodel to produce parallel flows and substrate concentration gradients in the pore structure. Due to substrate availability being affected by mass transfer, biomass grew in a single center mixing line of the micromodel. Biomass growth was only detected as finger-like aggregates down-gradient of silicon posts, not in pore throats where shear forces were not favorable. At the initial stages of the study, the line of biomass growth migrated slightly towards the PCE inlet. When PCE concentrations were increased from 0.2 mM to 0.5 mM, biomass growth migrated towards the lactate inlet instead. An analytical model was developed to explain the transverse hydrodynamics which caused the biomass to move towards lactate as the PCE concentration was increased. Fluorescent tracer experiments also showed that biomass growth blocked pores and created a higher velocity zone in the PCE-dominant half of the micromodel.

Referencing the micromodel design and analytical models developed by Nambi *et al.* (2003), Zhang *et al.* (2010) studied the degradation of (R)-2,4-DP by *D. acidovorans* MC1071 in micromodels with homogeneously distributed cylindrical posts and with aggregates of large and small cylindrical posts. (R)-2,4-DP and O_2 were injected into the micromodel to create a mixing gradient transverse to the direction of flow. Biomass growth was observed at the center mixing line of both pore structures. However, biomass growth in the homogenous pore structure was

uniform, while growth in the aggregated pore structure was slower, less dense, and had preference for zones in between aggregates. The homogeneous pore structure also yielded two times faster biomass growth and more (R)-2,4-DP degradation than the aggregate pore structure.

The long term objective of my project is to study the adaptive and competitive behavior of *D. acidovorans* strains in micromodels. Zhang *et al.*'s work was important in establishing most of the methods for inoculating, growing, and observing *D. acidovorans* in micromodels. It also gave insight to the kind of biomass morphology we can expect in our future experiments, and how we can use these morphologies to develop different levels of substrate mass transfer in our micromodels with biomass growth.

3.2. GFP-LABELING OF BACTERIA TO MONITOR SPATIOTEMPORAL BIOACTIVITY

Green fluorescent protein (GFP) was first purified from jellyfish *Aequorea* in the 1960s. (Shimomura *et al.*, 1962), and in the 1990s it became employed as a marker of gene expression in other prokaryotic and eukaryotic cells (Chalfie *et al.*, 1994). Soon, many alternate forms of GFP were being developed for different, specific expression requirements in laboratory experiments (Tsien, 1998); my research will involve constructing two GFP plasmids for use in the study of *D. acidovorans* adaptation and competition in micromodels.

For GFP to be useful in our monitoring of bacterial strain in micromodels, GFP must be suitable for utilization in spatiotemporal bioactivity monitoring. GFP was utilized by Stenberg *et al.* (1999) to monitor the spatial patterns of growth, inactivation, and reactivation of *P. putida* biomass. An additional requirement of the GFP utilized by Stenberg *et al.* was that expressed GFP needed to have short half-lives to identify biomass inactivation; unfortunately traditional GFP markers have long lifetimes which can retain fluorescence abilities long after induction. To

aid Sternberg *et al.* in their research, Andersen *et al.* (1998), developed GFP derivatives where short peptide sequences were added at the C-terminal of A*equorea* GFP. The resultant GFPs were susceptible to indigenous proteases in *E. coli* and *P. putida*, resulting in GFPs with half-lives of 40 minutes to a few hours. Sternberg *et al.* employed the variant GFPs developed by Andersen *et al.* to monitor and distinguish fast and slow growing *P. putida* cells in biofilms growing on benzyl alcohol. Cells that grew slowly were revived by having their growth stimulated with more easily metabolizable carbon sources; successful monitoring of such regrowth suggested that unstable variants of GFP can be developed to be good spatial trackers of activity in individual cells, cell clusters, or microcolonies.

Two biomass systems are intended for study in our micromodel experiments; the first is biomass adaptation. For successful monitoring of biomass adaptation, I wanted to construct a GFP derivative that can distinguish biomass activity and inactivity to certain substrates the biomass does not have native activity towards. In a precedence of GFP expression controlled by genetic expression limited by substrate availability, Bagge *et al.* (2004) produced a reporter of *ampC* promoter with *gfp*(ASV) encoding an unstable form of GFP and used this reporter to tag cultures of *P. aeruginosa* to monitor the expression of *ampC* in biofilms. β-lactam antibiotic resistance in *Pseudomonas aeruginosa* is indicated by high level expression of AmpC β-lactamase. The β-lactam antibiotic, imipenem, was used to induce GFP expression of a biofilm of tagged *P. aeruginosa*. Data from Bagge *et al.*'s study showed that GFP was only induced along the peripheries of the biofilm even though the entire biofilm was shown to be physiologically active with an arabinose-inducible GFP marker. This study thus showed that GFP can indeed be used in identifying specific biomass activities which may be heterogeneously expressed in physical environments.

The second biomass system that I intend to study involves biomass competition. To accomplish this, it is intended that mixed colonies of herbicide degrading bacteria are grown and the competition of individual strains within the colony with each other will be monitored. Sometimes, bacteria cells can migrate in growth environments to find regions they can more easily survive in. It is unknown whether biomass migration will occur in our future competition experiments, but the use of GFP to distinguish the movement of individual strains in mixed colonies has been shown by Tolker-Nielsen *et al.* (2000). In their study, Tolker-Nielsen *et al.* monitored growing cultures of *Pseudomonas* sp. B13 and *P. putida* OUS82 tagged with GFP and *Discosoma* sp. tagged with red fluorescent protein in a flow chamber with confocal scanning laser microscopy. Initially, separate red and green fluorescence microcolonies were observed. Eventually, distinct microcolonies of the green and red cultures began to grow within the colonies of the other color, suggesting that bacteria moved between the microcolonies. The successful distinction of specific colonies by Tolker-Nielsen *et al.* gives confidence that our monitoring of specific GFP-labeling *D. acidovorans* strains will also permit the distinction of individual bacterial strains in our micromodels.

CHAPTER 4: METHODOLOGY

4.1. EQUIPMENT STERILIZATION

Most laboratory equipment purchased unsterile was sterilized by 20-minute, 121°C gravity or liquid autoclave cycles. Glass containers were loosely capped or covered with aluminum foil, while small items such as microfuge tubes, pipette tips, syringe filters, or wooden sticks were placed in closable containers before autoclaving.

4.2. SOLUTION PREPARATION

All solutions were sterilized by 20-minute, 121°C liquid autoclave cycles and stored at room temperature unless otherwise stated.

4.2.1. HERBICIDE SOLUTIONS

50 mM herbicide solutions were prepared by dissolving 250 mg of (R)-2,4-DP (Prior to February 2011 – Riedel-de Haën Cat#: 36770; After February 2011 – Fluka Analytical Cat#: 31237), 2,4-DP (Sigma-Aldrich Cat: 08606EC), or 2,4-D (Sigma-Aldrich Cat: D70724-100G) in 21.27 mL of 100 mM NaOH solution. These solutions were then transferred to a general-use syringe and filtered through a 0.2 μm acetate-membrane filter (Nalgene Cat#: 190-9920) into a sterile 50 mL centrifuge tube (Sarstedt Inc. Cat#: 02-547-004). Sterilized herbicide solutions were stored at 4°C.

4.2.2. LURIA BERTINI MEDIA

Luria Bertini (LB) media was prepared by dissolving 20 g of LB Miller powder (Fisher Scientific Cat#: BP1426-2) in 1 L of deionized water. 100 mL aliquots of LB solutions were prepared in 200 mL glass bottles then immediately sterilized.

4.2.3. MINIMAL MEDIA

Liquid minimal media (Min) was prepared following procedures outlined in Müller, 1999. Mineral salt solution (MSS) and trace-element solution (TES) were prepared separately, then 999-parts of MSS and 1-part of TES were mixed together. MSS was prepared by dissolving 760 mg of NH_4Cl, 340 mg of KH_2PO_4, 485 mg of K_2HPO_4, and 18.12 mg of $CaCl_2 \cdot 2H_2O$ in 1 L of deionized water. The solution was adjusted to pH 8.5 with additional drops of 10 M NaOH solution and then sterilized. TES was prepared by completely dissolving, one-by-one, 4980 mg of $FeSO_4 \cdot 7H_2O$, 7850 mg of $CuSO_4 \cdot 5H_2O$, 615 mg of $MnSO_4 \cdot H_2O$, 440 mg of $ZnSO_4 \cdot 7H_2O$, 252 mg of $Na_2MoO_4 \cdot 2H_2O$, and 71,200 mg of $MgSO_4 \cdot 7H_2O$ in each 1L of 1N H_2SO_4. Since the volume of TES prepared was typically low, it was sterilized through 0.2 μm acetate-membrane filters. MSS and TES were only mixed to prepare Min after the MSS had time to cool after being removed from autoclave.

4.2.4. WORKING ANTIBIOTIC SOLUTION

Kanamycin and carbenicillin working solutions were prepared as 50 mg/mL solutions. Kanamycin powder (Fisher Bioreagents Cat: BP906-5) or carbenicillin disodium salt (Sigma Aldrich Cat#: C1389-5G) was dissolved in nanopure water and sterilized through 13 mm 0.22 μm-pore PTFE filters (Fisher Scientific Cat#: 09-720-7) into sterilized 1.5 mL polypropylene microfuge tubes (Fisher Scientific Cat#: 05-408-129). Sterilized antibiotic solutions were stored frozen at -20°C. The final concentration of kanamycin and carbenicillin for cell growth or maintenance was always 50 μg/mL and 100 μg/mL, respectively; cultures with both kanamycin and carbenicillin resistances were always grown on 50 μg/mL of kanamycin.

4.2.5. 20 mM PHOSPHATE BUFFER

Phosphate buffer at 20 mM (51% NaH_2PO_4 and 49% Na_2HPO_4) was prepared by adding the appropriate amounts of salt to nanopure water and then filtering through 0.2 μm filters and degassing through a sonicator under vacuum. Phosphate buffers were used as part of an HPLC mobile phase, and thus did not have to be sterilized.

4.2.6. SOC MEDIA

SOC media was prepared according to Section 5.3 of the *MicroPulser Electroporation Apparatus Operating Instructions and Applications Guide.* 2 g Bacto tryptone, 0.5 g Bacto yeast extract, 0.0584 g of NaCl, 0.0186 of KCl, 0.0952 g of $MgCl_2$, 0.1204 g of $MgSO_4$, and 0.3603 g of glucose were dissolved in 100 mL of distilled water in a 200 mL glass bottle.

4.2.7. TAE BUFFER

50× TAE buffer stock solutions were prepared with 121.0 g Tris base, 28.55 mL glacial acetic acid, and 9.305 g $Na_2EDTA•2H_2O$ dissolved in 500 mL of distilled water. TAE buffers were not sterilized.

4.2.8. DAPI SOLUTIONS

DAPI stock solution was prepared by dissolving solid DAPI powder (MP Biomedicals, Cat#: 157574) in water to a concentration of 5 mg/mL and stored at -20°C. DAPI working solution was prepared by diluting DAPI stock solution to 2 μg/mL with PBS and was stored at 4°C. All DAPI solutions were protected from light by aluminum foil.

4.3. PLATE PREPARATION

4.3.1. LB MEDIA PLATES

LB media plates were prepared by mixing 7.5 g of Bacto agar (BD Cat#: 214010) to 500 mL of LB before autoclaving; please note that Bacto agar will not dissolve in LB until autoclaved. After autoclaving, the media was momentarily swirled to evenly mix the dissolved agar throughout the LB media, and then the media was poured into approximately 22-25 100×15 mm sterile polystyrene Petri dishes (Fisher Scientific Cat#: 0875712). These plates were allowed to solidify at room temperature overnight, then stored closed and sealed with parafilm at 4°C.

4.3.2. MINIMAL MEDIA PLATES

Min plates were prepared by adding 7.5 g of Bacto agar to approximately 499.5 mL of pH-adjusted liquid MSS before autoclaving. After autoclaving and cooling the MSS, 0.5 mL of TES was added to the MSS, followed by 50 mM herbicide solution to reach a final herbicide concentration of 400 mg/L. The media was poured into approximately 22-25 100×15 mm sterile polystyrene Petri dishes.; these plates were allowed to solidify at room temperature overnight then stored closed and sealed with parafilm at 4°C.

4.3.3. ANTIBIOTIC MEDIA PLATES

LB plates with kanamycin were prepared by pipetting kanamycin solution into autoclaved and cooled LB/agar solutions to make a final kanamycin concentration of 50 μg/mL.

Min plates with kanamycin were prepared by pipetting 20 μL of sterilized distilled water and 20 μL of working kanamycin solution onto a solidified media plate, and then evenly spreading the water/antibiotic solution over the top of the media plate with a flame-sterilized glass spreader. LB or Min plates with carbenicillin were prepared similarly with 40 μL of working carbenicillin solution instead of water/kanamycin solution.

4.4. CELL GROWTH AND MAINTENANCE

4.4.1. *Escherichia coli*

Escherichia coli S17-1 λ-pir was received from Dr. Michael G. Thomas at the University of Wisconsin (Simon *et al.*, 1983). *E. coli* DH5α was received from the Cell Media Facility of the School of Chemical Sciences at The University of Illinois at Urbana-Champaign.

Frozen *E. coli* cultures were prepared by pipetting 0.8 mL of liquid *E. coli* culture into 0.8 mL of sterilized 80% glycerol in 2 mL capped polypropylene tubes. These stocks were stored frozen at -80°C.

Liquid *E. coli* cultures were prepared as 1-10 mL aliquots. 1-2 mL cultures were prepared in sterilized 10mL glass test-tubes, while 5-10 mL cultures were prepared in sterilized 125 mL Erlenmeyer flasks. Antibiotic solutions were first pipetted into the appropriate glassware, followed by LB liquid media. The prepared media was then inoculated with single colonies picked from media plates or a frozen stock with a sterilized wooden stick. Inoculated media were incubated at 37°C, with shaking at 200-225 RPM for 10-12 hours.

Active cultures of *E. coli* were maintained on LB media plates with corresponding antibiotics. Plates of streaked cultures were prepared by picking and streaking cells from other media plates or liquid culture. Plates were incubated at 37°C for 16-24 hours then stored at 4°C for 4-6 weeks, after which a new culture plate was prepared.

E. coli electrocompetent cells were grown for use in transformation experiments following the protocol outlined in Section 5.1 of the *MicroPulser Electroporation Apparatus Operating Instructions and Applications Guide*. 7 mL of LB was pipetted into a 125 mL Erlenmeyer flask and inoculated with *E. coli* from plated cultures, then incubated for 24 hours at 37°C, shaking at

200 RPM. Four aliquots of 125 mL LB were then poured into four sterilized 500 mL Erlenmeyer flasks with sidearms and each LB solution was inoculated with 1.25 mL of the liquid *E. coli* culture. The cultures were again grown at 37°C, shaking at 200 RPM. Readings of light absorbance at 600 nm by the samples were taken every hour. Once cell density in the cultures was enough to cause a 0.5-0.7 absorbance reading (approximately 5 hours), the cultures were cooled on ice for 50 minutes. Every two samples were combined in 500 mL centrifuge bottles and the samples were centrifuged at 4°C at 2831 rcf for 15 minutes. Supernatant from centrifugation were discarded and cell pellets were washed with 250 mL of sterilized 10% ice-cold glycerol and centrifuged again. A second wash with 125 mL sterilized 10% ice-cold glycerol was performed before the cell pellets were resuspended in 20 mL of 10% ice-cold glycerol and transferred to a smaller centrifuge tube for a third centrifugation wash. The final cell pellet was re-suspended in 2 mL of 10% ice-cold glycerol and 0.5 mL aliquots of this cell suspension were pipetted into sterilized 2 mL capped tubes. Cell suspensions were quickly frozen in a dry-ice/alcohol bath and immediately stored at -80°C until use.

4.4.2. *Delftia acidovorans* MC1 AND MC1071

Delftia acidovorans MC1 (Müller *et al.*, 1999) and its derivative MC1071 (Müller, unpublished) were received from Dr. Roland Müller at the Helmholtz Centre for Environmental Research.

Frozen MC1 and MC1071 stocks were prepared by pipetting 0.8 mL of liquid *D. acidovorans* culture into 0.8 mL of sterilized 80% glycerol. These stocks were stored frozen at -80°C.

Liquid *D. acidovorans* cultures were prepared as 10 mL aliquots. MC1 was grown with 2,4-DP, while MC1071 was grown with (R)-2,4-DP acting as the carbon source. Antibiotic solutions were first pipetted into a 125 mL Erlenmeyer flask, followed by 25 mg/L of herbicide, then finally 10 mL of LB liquid media. The prepared media was then inoculated with 5-8 colonies picked from media plates. Inoculated media were incubated at 30°C, shaking at 150-200 RPM; MC1071 cultures were grown for 14-16 hours while MC1 cultures were grown for 10-12 hours. These cultures were then pipetted into sterile 15 mL polypropylene centrifuge tubes (Corning Inc. Cat#: 430766) and spun down in an Eppendorf 5416 Centrifuge at 2375 rcf for 10 minutes. The supernatant was discarded and 5 mL of Min was added to the top of the culture pellet. The pellet was resuspended into the Min by being pipetted in and out of a sterilized 5 mL pipette tip several times before being transferred to a fresh, sterilized 125 mL Erlenmeyer flask. Next, 25 mg/L of herbicide was added to the culture solution, followed by an additional 5 mL of Min. The culture was incubated for 3 hours at 30°C, shaking at 150-200 RPM, with samples taken before and after to determine the solution's starting and ending herbicide content. If herbicide was completely degraded, 200 mg/L of herbicide was added to the culture, and the culture was grown for an additional 12 hours at 30°C, shaking at 150-200 RPM; samples were once again taken before and after this incubation period to determine the solution's starting and ending herbicide content. If a second 12-hour growth period in Min was required, another 200 mg/L of herbicide was added after the first 12-hour growth period.

Plated active cultures of *D. acidovorans* were maintained on Min plates with corresponding antibiotics and herbicide. Plates of streaked cultures were prepared by picking and streaking 5-8 colonies from other media plates or from liquid culture. Plates were incubated at 30°C for 3-7

days, then cultures were stored at 4°C for 4-6 weeks, after which a new culture plate was prepared.

4.5. HERBICIDE CONCENTRATION BY HPLC ANALYSIS

4.5.1. SAMPLE PREPARATION

HPLC sample vials were prepared by placing a 5×30 mm, 0.250 mL flat bottom glass insert (Laboratory Supply Distributors Corp Cat#: 20870-530) into a National Scientific assembled 2 mL sample vial (Fisher Scientific Cat#: C4013-415A). HPLC samples were prepared by pulling 0.3 mL of sample through 1 mL general use syringes (BD Cat#: 309602), then filtering through 13 mm, 0.22 μm-pore PTFE filters with a 23 G×1 in needle tip (BD Cat#: 305193) into a prepared sample vial. Sample vials were then closed with screw-thread caps (National Scientific Cat#: 21200J-08) fitted with Teflon septa (National Scientific Cat#: C4013-10).

4.5.2. SHIMADZU HPLC SYSTEM OPERATION

The Shimadzu HPLC system consists of a SIL-20A Auto Sampler, LC-20AT Solvent Delivery Module, CTO-10AS Column Oven, SPD-M20A UV/VIS Photodiode Array Detector, and CBM-20A System Controller and was operated from a Windows OS by the LCsolutions software. The HPLC column used was a silica-based Dionex Acclaim 120 C18 3 μm 120 Å reversed-phase C18 column.

HPLC analysis conditions were developed and provided by Changyong Zhang; the conditions were partially outlined in Zhang, 2010. During analysis, the HPLC column's operating temperature was set at 40°C. 15 mL/hr of degassed nanopure water, 15 mL/hr of 20 mM degassed phosphate buffer solutions, and 30 mL/hr of degassed HPLC-grade methanol (Fisher Scientific Cat#: AC61009-0040) were used as the mobile phase during operation. Each

sample was analyzed for 15 minutes. HPLC chromatograms of samples were blanked against a baseline of four nanopure water samples ran during each time the HPLC system was started up.

4.5.3. QUANTIFICATION OF HERBICIDE CONCENTRATIONS

Herbicide concentrations were analyzed on the 205 nm absorbance spectra versus time. Spectra and a list of integrated peak areas from samples were exported to text-files and analyzed in Microsoft Excel. Peak areas were treated as being directly correlated to solute concentrations. 2,4-D peaks typically showed up at 4 minutes of runtime, while 2,4-DP peaks showed up at 5-7 minutes of runtime. Sample HPLC spectrums of 2,4-D and (R)-2,4-DP are shown in Figure 5.

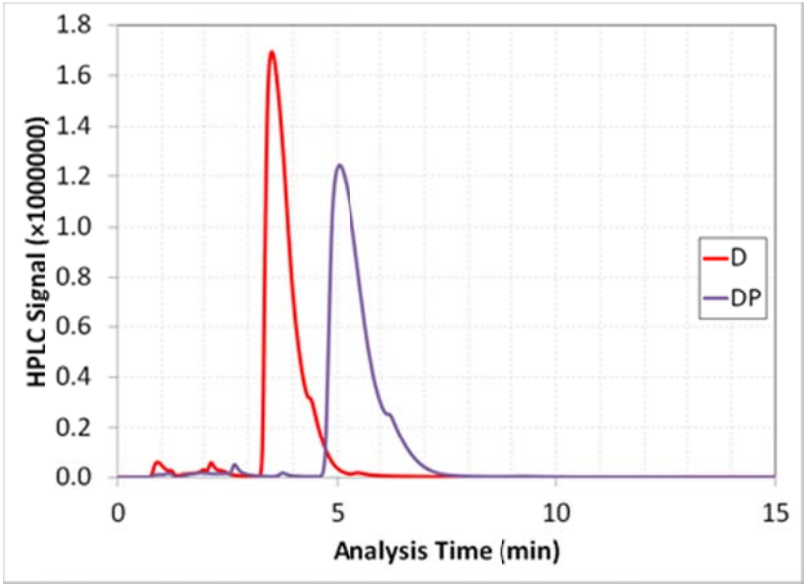

Figure 5: HPLC Spectrum of 496 mg/L 2,4-D and 503 mg/L (R)-2,4-DP Standard Solutions

4.5.4. CALIBRATION

~500 mg/L stock solutions of 2,4-D and (R)-2,4-DP were prepared volumetrically in 100 mM NaOH solution. The stock solutions, as well as 1:2, 1:4, 1:8, and 1:16 dilutions of the stocks were then analyzed in the Shimadzu HPLC. Linear calibration curves of signal peak area versus

herbicide concentration for each herbicide were then determined in Microsoft Excel with a forced zero-fit.

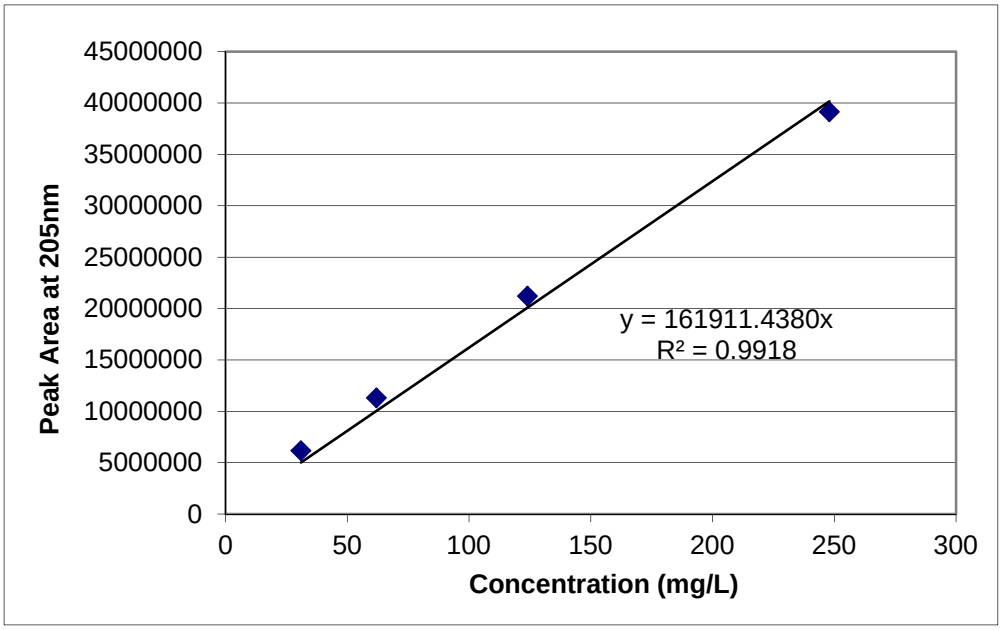

Figure 6: HPLC 2,4-D Calibration Curve

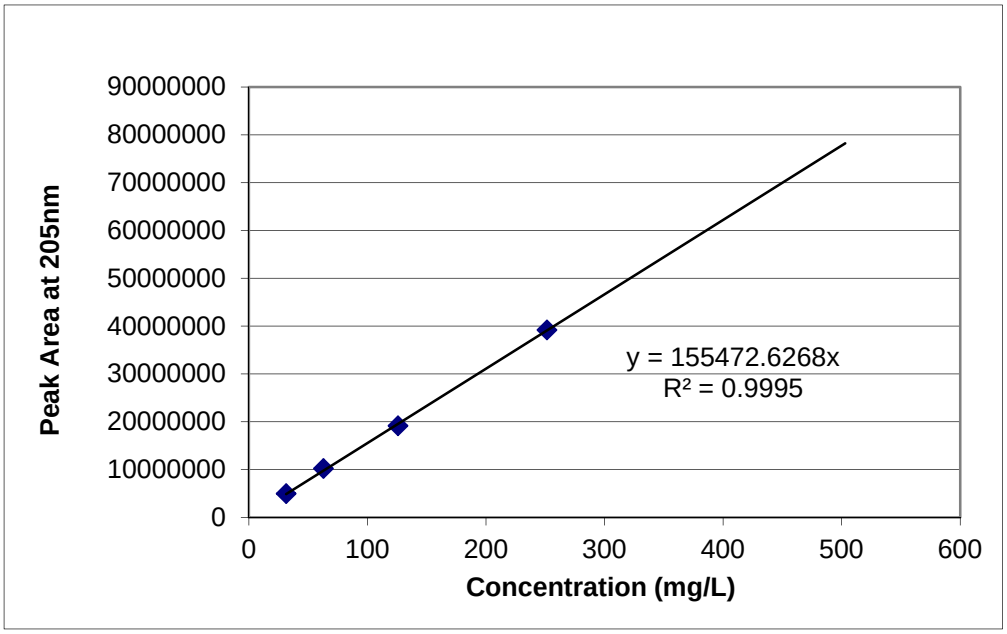

Figure 7: HPLC (R)-2,4-DP Calibration Curve

From the calibration curves shown in Figure 6 and Figure 7, relations between HPLC signal peak areas and herbicide concentrations were determined.

$$[2,4\ D] = (Peak\ Area)_{signal} / 161911.4380$$

$$[2,4\ DP] = (Peak\ Area)signal / 15572.6268$$

4.6. QIAGEN PLASMID EXTRACTION

Plasmid samples were extracted using Qiagen Miniprep Kits, following protocols outlined by the manufacturer. Plasmids were typically extracted from 5 mL of *E. coli* liquid cultures grown for 14-16 hours just prior to extraction. Cell cultures were spun down in 1.5 mL microfuge tubes at 93000 rcf for 3 minutes (multiple decanting of supernatant and reloading of samples were required). In the final step, plasmid DNA was eluted with 50 μL of Buffer EB (10 mM Tris-Cl, pH 8.5). Plasmid DNA samples were stored at -20°C.

4.7. DNA CONCENTRATION DETERMINATION BY NANODROP ANALYSIS

Plasmid DNA concentrations were determined with a Thermo Scientific NanoDrop 1000 Spectrophotometer. The absorbance spectra of 2 μL DNA solution samples were compared to the absorbance spectra of 2 μL samples of nanopure water. The spectrophotometer's operating software automatically calculated the concentration of DNA in a solution based on its absorbance at 260 nm. DNA samples were considered pure when the absorbance ratio of 260/280 nm was ~1.8 and 260/230 nm was ~2.0-2.2. A significantly lower 260/280 nm ratio is

indicative of protein presence, while a lower 260/230 nm ratio indicates the presence of EDTA, carbohydrates, or phenols. Figure 8 shows an example of the results outputted by the NanoDrop system from a sample of plasmid DNA pKN*lacgfp*.

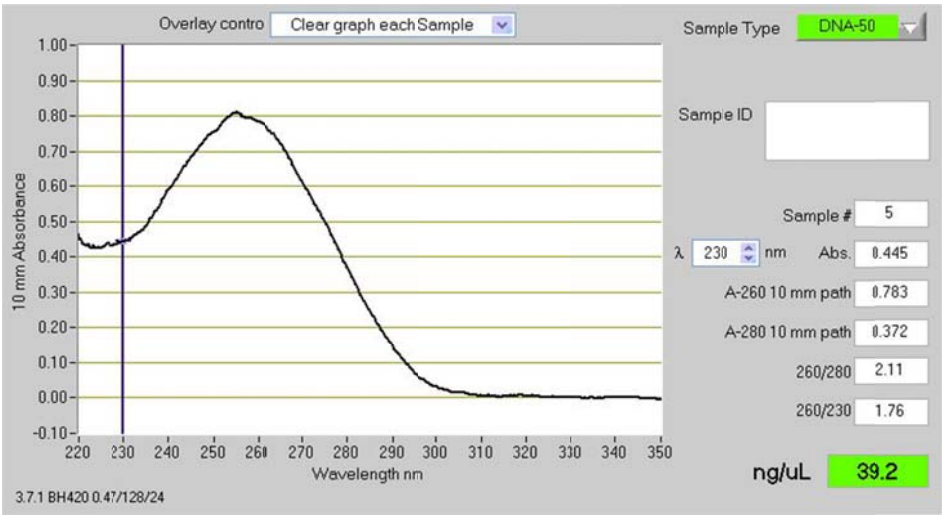

Figure 8: Sample Nanodrop Spectrum of pKN*lacgfp*

4.8. GEL ELECTROPHORESIS

DNA gel electrophoreses were most commonly performed on 1% agarose gels. For 20 mL gels, 0.2 g of agarose was mixed with TAE buffer in a 50 mL Erlenmeyer flask; double amounts were used for 40 mL gels. The flask and its contents were tared on an analytical balance. The agarose was melted by alternately being heated in a microwave and swirling within the flask. Heating continued until no gas bubbles were observed in the agarose solution, then distilled water was added to the flask to replace any water evaporated during the heating process. The solution was poured into a gel mold shown in Figure 9, a sample-well comb as partially submerged into the solution, and the gel was allowed to solidify at 4°C. During this time, 400 mL of TAE was also cooled at 4°C.

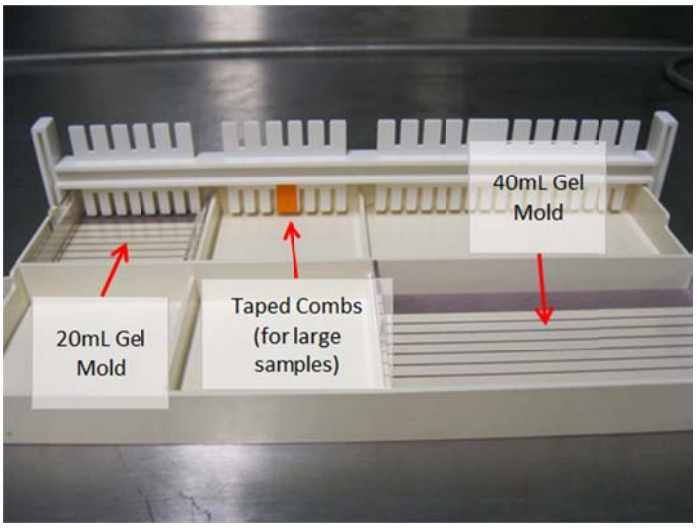

Figure 9: Electrophoresis Gel Mould

After the gel solidified, it was removed from the mould and placed in a Stratagene Joule Box electrophoresis chamber. Enough cooled TAE was added to submerge the gel by several millimeters. For each ≤10 µL DNA sample to be loaded, a 1 µL drop of 10× BlueJuice Loading Buffer (Invitrogen Cat#: 10816-015) was pipetted onto parafilm; larger DNA samples requiring loading buffer had BlueJuice added by an exact 9:1 sample:buffer ratio. DNA samples were pipetted onto a drop of loading buffer and thoroughly mixed by repeated pipetting. Each DNA sample with loading buffer was then loaded into a well within the agarose gel. The electrophoresis apparatus was then closed, its diodes attached, and turned on to apply current across the chamber. The voltage depended on the projected size of DNA samples to be tested; samples smaller than 1 kbp were typically ran at 50 V, while larger samples were ran at 100 V. Electrophoresis was terminated when the blue stain of the loading buffer had migrated 70-90% the length of the agarose gel.

4.8.1. PLASMID SIZE DETERMINATION

For plasmid size determination, agarose DNA Grade (Fisher Scientific Cat#: BP164-100) was used. DNA ladders (Invitrogen 1 kb DNA Ladder Cat#: 15615-016 or Promega Lambda DNA/HindIII Marker Cat#: G1711) were always loaded to the sample wells at one or both extremes of gels. After electrophoresis, the entire gel was stained, in darkness, for 20 minutes in a 1× SYBR Gold solution made of 10 μL 10000× SYBR Gold stock and (Invitrogen Cat#: S11494) and 100 mL of distilled water. After staining, gels were allowed to stand for 3-5 minutes before being illuminated by a Spectroline Ultraviolet Transillimiinator .Photos of illuminated gels were taken by a Kodak DC290 Camera with exposures ranging from 2-5 seconds.

4.8.2. DNA PURIFICATION FROM GEL SLICES

For DNA purification, LMP agarose (Promega Corporation Cat#: V2831) was used. DNA ladders were loaded to the extremes of the gel, and reference DNA samples were loaded just inside from the ladders. DNA samples to be purified were loaded into larger sample wells produced by taping several teeth of well combs together before setting the gels. After electrophoresis, the edges of the gel containing ladder and references were cut out with a metal spatula and stained, in darkness, for 20 minutes in 1× SYBR Gold solution. After staining, these gel fragments were allowed to stand for 3-5 minutes before being illuminated. Metal spatulas were used to make markings into where reference DNA fragments showed up. The entire gel was then pieced back together and the un-illuminated DNA sample was excised with a metal spatula. The agarose gel containing DNA sample was weighed and stored at -20°C in a pre-weighed 1.5 mL microfuge tube. Photos of the entire illuminated gel were then taken with exposures ranging from 2 – 5 seconds.

Agar*ACE* enzyme (Promega Cat#: M1741) was used, according to the protocols provided with the product, to purify DNA from excised pieces of agarose gel. Gels were melted at 65°C in a Fisher Scientific Dry-Bath Incubator (heating block) inside 1.5 mL microfuge tubes. After the gel samples had their agarose digested and washed off through centrifugation and decanting, the samples were reconstituted in 10-50 μL of Qiagen EB buffer. DNA samples were allowed at least 15 minutes to resuspend before being stored at -20°C or used in further experiments.

4.9. RESTRICTION ENZYME DIGESTS

Restriction enzymes were stored at -20°C and only defrosted on ice immediately before digest experiments. Digests were performed in sterile 1.5 mL microfuge tubes, in optimal buffer with 100% enzyme activity, or when 100% activity was not possible, for example in double digests, then at a minimum of 75% activity. Digest incubation temperatures and times as stated by enzyme providers were used. When needed, digest solutions were supplemented with Bovin serum albumin (BSA); in the case of double digests where only one of the enzymes require BSA, the solutions were still supplemented with BSA as the serum had no adverse effects on enzymes that did not require BSA.

Restriction Enzyme	**Supplier**	**100× BSA Required**	**Buffer**	**Incubation Condition**	**Heat Inactivation**
*Eco*RI (10 u/μL)	Invitrogen Corporation	No	NEBuffer 1 (100%) NEBuffer 2 (100%) NEBuffer 3 (100%) NEBuffer 4 (100%)	37°C 1 hour	65°C 20 minutes
*Hinc*II (10 u/μL)	New England Biolabs	Yes	NEBuffer 1 (75%) NEBuffer 2 (100%) NEBuffer 3 (100%) NEBuffer 4 (100%)	37°C 1 hour	65°C 20 minutes
*Hin*dIII (10 u/μL)	Promega Corporation	No	NEBuffer 2 (100%)	37°C 1 hour	65°C 20 minutes
*Kpn*I (10 u/μL)	New England Biolabs	Yes	NEBuffer 1 (100%) NEBuffer 2 (75%)	37°C 1 hour	None
*Nco*I (10 u/μL)	New England Biolabs	No	NEBuffer 1 (100%) NEBuffer 2 (100%) NEBuffer 3 (100%) NEBuffer 4 (100%)	37°C 1 hour	65°C 20 minutes
*Nsi*I (10 u/μL)	New England Biolabs	No	NEBuffer 2 (75%) NEBuffer 3 (100%)	37°C 1 hour	80°C 20 minutes
*Pst*I (10 u/μL)	Invitrogen Corporation	Yes	NEBuffer 1 (75%) NEBuffer 2 (75%) NEBuffer 3 (100%)	37°C 1 hour	80°C 20 minutes
*Sac*I (10 u/μL)	Promega Corporation	Yes	NEBuffer 1 (100%) NEBuffer 4 (100%) Buffer J (100%)	37°C 1 hour	65°C 20 minutes
*Sma*I (10 u/μL)	Invitrogen Corporation	No	NEBuffer 4 (100%)	25°C 1 hour	65°C 20 minutes
*Xcm*I (5 u/μL)	New England Biolabs	No	NEBuffer 2 (100%)	37°C 1 hour	65°C 20 minutes

Table 1: Restriction Enzymes Employed in Study

4.10. PLASMID CONSTRUCTION

All plasmids either used or constructed in this study are listed in Table 2. Three plasmids used in this study were provided by outside sources. pBBR1MCS-2 (Kovach *et al.* 1995), a broad-host-range (bhr) cloning vector encoding kanamycin-resistance (Km^R) was provided courtesy of The Department of Biology & Geology at Baldwin Wallace College. pBBR1MCS-2 is a derivative of pBBR1MCS, a carbenicillin-resistant bhr plasmid with multiple cloning sites (MCS) within a *lac*Zα gene (Kovach *et al.*, 1994). pAK*gfp*1 (Karsi and Lawrence, 2007) was provided by Addgene. (Addgene plasmid 14076). The *tfdC* promoter utilized in our studies was synthesized according to known sequences (Müller, unpublished; Schleinitz, 2004) by BlueHeron Biotechnology with *XcmI* and *KpI* restriction sites added upon synthesis. The gene was provided in alternate vector pUC.

Plasmid	**Reference**
pBBR1MCS-2	Kovach *et al.*, 1995
pAK*gfp*1	Karsi and Lawrence, 2007
pUC (with *tfdC*)	Schleinitz, 2004
pAG408	Suarez *et al*., 1997
pKN*lacgfp*	
pKN*tfdgfp*	

Table 2: Plasmids Used in Study

4.10.1. CONSTITUTIVE PLASMID pKN*lacgfp*

Plasmid pKN*lacgfp*, which provides kanamycin resistance and expresses green fluorescent protein using of the *lac* promoter, is composed of plasmid pBBR1MCS-2 (Kovach *et al.*, 1995) and an inserted fragment from pAK*gfp*1 (Karsi and Lawrence, 2007). Schematic representation of the construction of pKN*lacgfp* is shown in Figure 10.

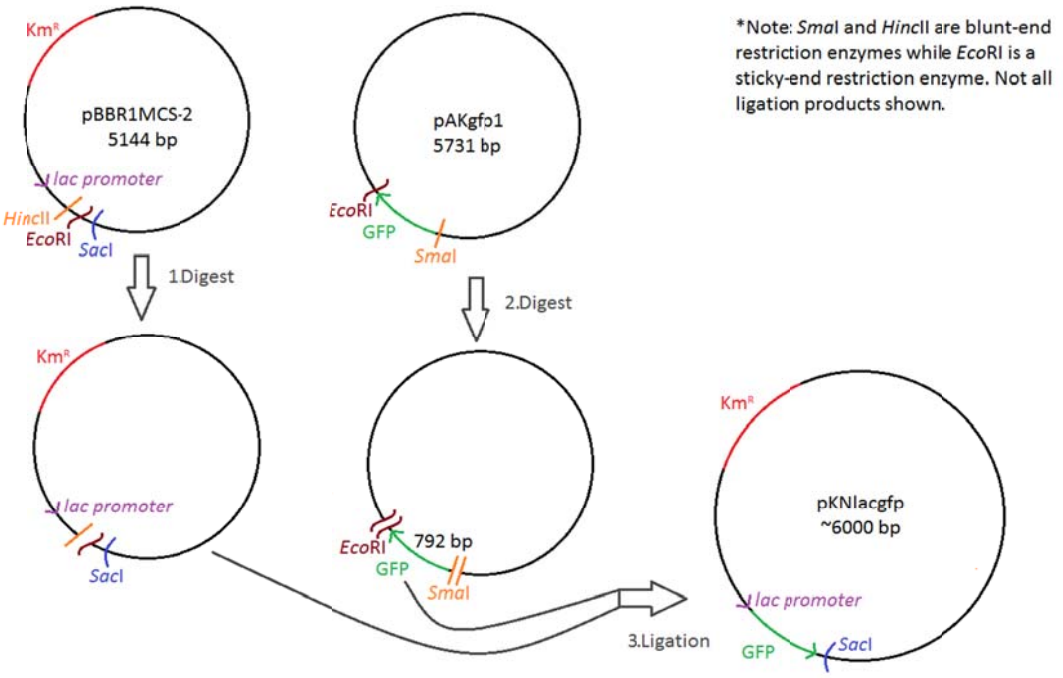

Figure 10: Schematic Representation of pKN*lacgfp* Construction (Excision of *gfpmut3a* from pAK*gfp*1 and Insertion into Km^R pBBR1MCS-2)

To construct pKN*lacgfp*, the appropriate gene fragments had to be cut from each original plasmid by double restriction enzyme digests; temperatures and incubation times are listed in Table 1. In step 1 (Figure 10), 823.2 ng of pBBR1MCS-2 (102.9 ng/µL × 8 µL) was digested with 3 units of *Eco*RI and 6 units of blunt-ended restriction enzyme *Hinc*II in NEBuffer 3 in a 10 µL digest solution, followed by heat inactivation. In parallel, step 2 of Figure 10 was performed, where 1302.9 ng of pAK*gfp*1 (8.6 µL × 151.5 ng/µL) was digested with 3 units of *Eco*RI in NEBuffer 4 in a 10 µL digest solution, followed by heat inactivation. Next, 6 µL of the digest solution was digested with 1.8 units of blunt-ended restriction enzyme *Sma*I, followed by heat inactivation.

In step 3 of Figure 10, the plasmid fragments were then pieced together with T4 DNA Ligase (Promega Corporation Cat#: M180A). 2.67 µL of pAK*gfp*1 digest and 1.23 µL of

pBBR1MCS-2 digest were mixed and supplemented with 4.8 μL of distilled water, 0.3 μL (0.9 units) of T4 DNA Ligase, and 1 μL of T4 Ligase Buffer (Promega Corporation Cat#: C126A). The solution was incubated at 4°C for 24 hours. The ligation solution was electroporated into *E. coli* S17-1 λ-pir, as outlined in Section 4.11.

4.10.2. INDUCIBLE PLASMID pKN*tfdgfp*

Plasmid pKN*tfdgfp*, which provides expresses green fluorescent protein by way of the *tfdC* promoter and kanamycin resistance, is composed of plasmid pKN*lacgfp* and an inserted fragment of *tfdC* promoter. Schematic representation of the construction of pKN*tfdgfp* is shown in Figure 11.

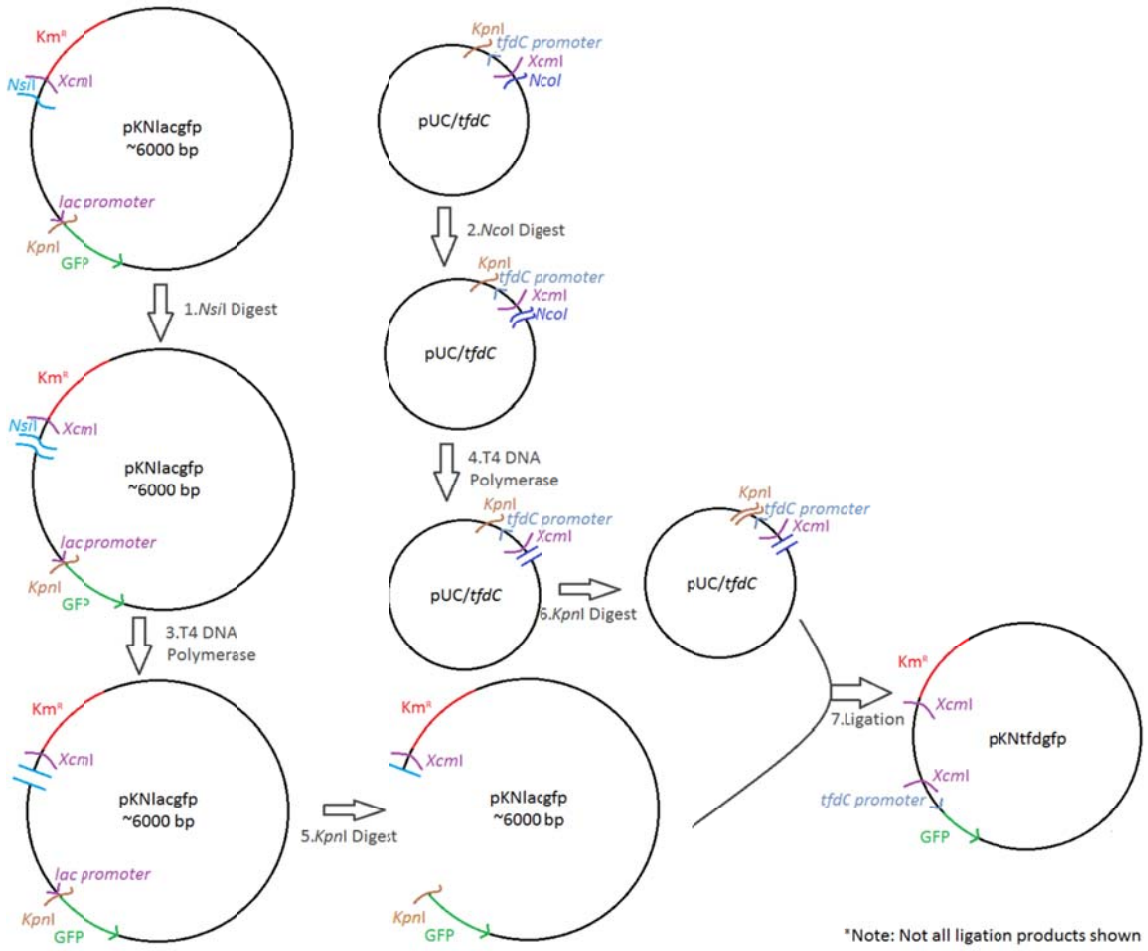

Figure 11: Schematic Representation of pKNtfdgfp Construction (Replacement of *lac* promoter by *tfdC* promoter in pKNlacgfp)

The promoter was constructed according to Schleinitz, 2004, and incorporated into plasmid pUC by BlueHeron Biotechnologies Inc (CCN: 75343). The *tfdC* promoter sequence is shown in Figure 12.

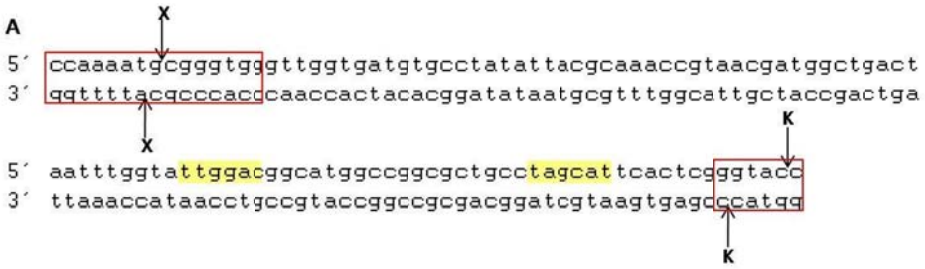

Figure 12: *tfdC* Promoter Sequence

To construct pKN*tfdgfp*, the *lac* promoter had to be separated from pKN*lacgfp* and the *tfdC* promoter from pUC/*tfdC* with double restriction enzyme digests; temperatures and periods at which restriction enzyme digests were performed are listed in Table 1. In step 1 of Figure 11, 1693.18 ng of pUC/*tfdC* (8.65 µL × 189.5 ng/µL) was digested with 3.5 units of *Nco*I in NEBuffer 2 in a 10 µL digest solution, with no heat inactivation. In parallel, step 2 of Figure 11 was performed, where 1695.40 ng of pKN*lacgfp* (39.2 ng/µL × 43.25 µL) was digested with 17.5 units of *Nsi*I in a 50 µL digest solution, with no heat inactivation. 1 µL was taken out of each digest solution in case controls needed to be performed.

To create compatible ends in the digested plasmid fragments for ligation, T4 DNA Polymerase (New England Biolabs Cat#: M0203S) was used to turn the digested *Nco*I and *Nsi*I sites into blunt ends. In step 3 of Figure 11, 1.65 units of T4 DNA Polymerase, approximately 5 µg of dNTP, additional NEBuffer 3, and additional BSA were added to the pKN*lacgfp* digest solutions. In step 4 of Figure 11. 1.47 units of T4 DNA Polymerase, approximately 1 µg of dNTP, additional NEBuffer 2, and additional BSA were added to the pUC/*tfdC* digest solution. Both polymerase reactions were performed at 12°C for 15 minutes. The reaction solutions were then immediately loaded onto EconoSpin all-in-1 mini spin columns (elution colums; Epoch Life

Science Cat#: 1910-250). Polymerase reaction chemicals were washed through the column with 0.75 mL of Qiagen buffer PE, then the plasmids were eluted into sterile 1.5 mL centrifuge tubes with 50 μL of Qiagen buffer EB.

In steps 5 and 6 of Figure 11, 42.75 μL of each plasmid solution was digested with 17.5 units of *Kpn*I in NEBuffer 1 in a 50 μL digest solution. Each plasmid was once again purified through elution columns with a 0.75 mL Qiagen buffer PE wash and 50 μL of Qiagen buffer EB elution. The concentrations of pUC/tfdC and pKNlacgfp solutions were determined to be 7.5 ng/μL and 12 ng/μL, respectively by nanodrop analysis.

The two digests were ligated together in step 7 of Figure 11. Two ligation solutions were prepared with the purified plasmid solutions. Ligation 1 consisted of 2.23 μL of pUC/*tfdC* digest, 6.47 μL of pKN*lacgfp* digest, 0.3 μL (0.9 units) of T4 DNA Ligase, and 1 μL of T4 Ligase Buffer. Ligation 2 consisted of 3.8 μL of pUC/*tfdC* digest, 4.9 μL of pKN*lacgfp* digest, 0.3 μL (0.9 units) of T4 DNA Ligase, and 1 μL of T4 Ligase Buffer. Both ligation were performed at 4°C for 18 hours. The ligation solutions were electroporated into *E. coli* S17-1 λ-pir, as outlined in Section 4.11.

4.11. PLASMID ELECTROPORATION

Cell transformations by electroporation were performed with a Bio-Rad MicroPulser Electroporation Apparatus (Bio-Rad Cat#: 165-2100). Electroporation protocols can be found in Section 5 of the *MicroPulser Electroporation Apparatus Operating Instructions and Applications Guide*. 0.1 cm electroporation cuvettes (Bio-Rad Laboratories Cat#: 165-2089)

were selected for use in our transformation experiments. Transformed cultures were plated on LB plates with antibiotic selectivity and incubated overnight at 37°C.

After incubation, the number of colonies on selective plates were counted and recorded. Individual colonies were chosen and streaked onto LB plates with antibiotics. To determine the nature of the plasmid which transformed selected colonies, plasmids were extracted from streaked cultures via Qiaprep plasmid extraction, digested with restriction enzymes, and then analyzed by gel electrophoresis.

4.12. CELL CONJUGATION

Plasmids were introduced into *D. acidovorans* recipient cultures by conjugation experiments with *E.* coli S17-1 λ-pir donor cultures housing the plasmids. To prepare bacterial cultures needed in conjugation experiments, 10 mL liquid cultures of *D. acidovorans* MC1071 and *E. coli* S17-1 λ-pir with plasmid were grown such that the two cultures would mature at the same time. After culture maturation, 1mL of MC1071 was subcultured into 9 mL of LB with 25 mg/L of (R)-2,4-DP, while 0.1 mL of S17-1 λ-pir was subcultured into 9.9 mL of LB with appropriate antibiotic. Subcultures were incubated for 3 hours at appropriate temperatures. 1 mL of each subculture was centrifuged at 16100 rcf for 2 minutes, with the pellets being washed once with 500 μL of LB and spun again. The two pellets were then resuspended together in 250 μL of LB, votexed for 1-2 seconds, and spread on approximately the inner-half diameter of an LB plate.

After overnight incubation (12-24 hours) at 30°C, biomass on the plates were scraped and resuspended into 2 mL of LB. 1×20 μL and 2×200 μL aliquots were plated onto Min plates with selective antibiotics according to the plasmid being transferred. These plates were incubated at

30°C for 3 – 7 days, after which individual colonies were picked and restreaked on Min plates with selective antibiotics and grown again for 3 – 7 days.

Plasmids were usually extracted from liquid cultures of isolated strains, digested by restriction enzymes, and then ran in gel electrophoresis to determine plasmid identity.

4.13. SPECTROPHOTOMETRIC DETECTION OF FLUORESCENCE

The relative fluorescence intensities of different bacterial strains were quantified with a Molecular Devices SpectraMax Gemini Spectrophotometer. For *E. coli* strains, liquid cultures were grown at 37°C in LB for 12-14 hours. *E. coli* cultures requiring IPTG to express fluorescence were supplemented with 1 mM of IPTG for the final 2 hours of incubation. For *D. acidovorans* strains, liquid cultures were grown at 30°C in LB for 14-16 hours, spun down and resuspended for growth in Min with 25 mg/L of herbicide for 3 hours, then grown still in the same solution with 200-400 mg/L of herbicide for 12 hours. After growth, cultures were spun down and resuspended in an equal volume of Min as the original cultures. For every culture to be tested, 3×200 μL resuspended samples were pipetted onto a standard black 96-well plate (Corning Incorporated Cat#: 3694). Plates were read in the spectrophotometer with excitation/emission wavelengths of 485/538 nm and auto cut-off wavelength of 530 nm at 37°C. Average fluorescence signal and standard deviations were calculated from the 3 samples in Microsoft Excel. [Fluorescence of individual bacterial strains were measured repeatedly to ensure reproducibility.]

4.14. MICROSCOPIC DETECTION OF FLUORESCENCE

Liquid cultures of tested strains were grown with selective antibiotics as described. Cultures requiring IPTG to express fluorescence were supplemented with 1 mM of IPTG (from

100 mM IPTG solutions) for the final 2 hours of incubation. 50-100 μL samples of liquid cultures were pipetted into microscope slide sampling ports and air-dried covered from light (~30 – 60 minutes). Enough DAPI working solution was added to cover each sampling port and incubated covered from light for ~5 minutes. DAPI solutions were pipetted from the slides and the slides were gently rinsed with distilled water. One-drop of Citifluor was added to each sample, then a microscope slide cover was placed on top of the samples and seal with nail polish.

A Zeiss Axioskop Routine Microscope was used to image cultures at a 100× magnification, while a Leica DMI 5000M microscope was used at 10-40× magnification. To image samples with the Axioskop, Immersol immersion oil (Carl Zeiss Cat#: 518F) had to be added onto the microscope slide cover on top of each sample. Cells were identified by the DAPI filter of the Axioskop, while white-light images were obtained by the Leica microscope. Green-fluorescent cultures were imaged through the FITC filter of either microscope.

CHAPTER 5: RESULTS AND DISCUSSION

5.1. CONSTRUCTION OF CONSTITUTIVE PLASMID pKN*lacgfp*

The insertion of *gfpmut3a* from pAK*gfp*1 into Km^R plasmid pBBR1MCS-2 is described in section 4.10.1. The constructed constitutive plasmid pKN*lacgfp* was suspected to be within the ligation product of 4.10.1. In order to screen for the correct plasmid from the ligation product, the ligation product was electroporated into a competent culture of S17-1 λ-pir. After electroporation, the S17-1 λ-pir culture was suspended in SOC media. One aliquot of 20 μL and two aliquots of 200 μL of the culture were spread on 3 separate LB plates with kanamycin. After overnight growth at 37°C, 28 cultures were identified. 21 of these cultures were restreaked onto fresh LB plates with kanamycin and grown overnight at 37°C.

A plasmid extraction was performed on each of these 21 cultures. Single restriction enzyme digests and gel electrophoresis were performed on these plasmid extracts to determine, by size, whether any of the extracted plasmids had received the *gfpmut3a* insert. Plasmid extracts were digested with restriction enzyme *Sac*I prior to the gel electrophoresis shown in Figure 13.

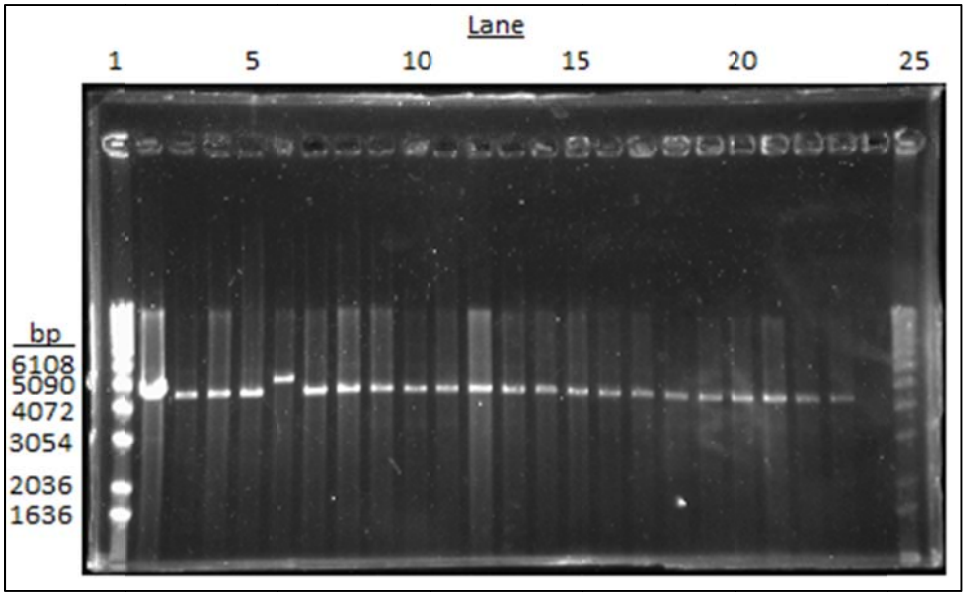

Figure 13: Gel Electrophoresis Distinction of Plasmids Yielded from Ligation of *gfpmut3a* to pBBR1MCS-2 – Lanes 1 and 25: Invitrogen standard 1 kB DNA Ladders; Lane 2: *Sac*I digested pBBR1MCS-2; Lanes 3-23: *Sac*I digested plasmid extracts from S17-1 λ-pir cultures isolated from electrophoresis

Since *Sac*I is a unique restriction enzyme site on pBBR1MCS-2 and is not present on the *gfpmut3a* fragment, *Sac*I should perform a single cut on both pBBR1MCS-2 and any plasmids where *gfpmut3a* had been inserted. pBBR1MCS-2 (Lane 2 of Figure 13) is a 5144 bp plasmid and as expected lies next to the 5090 bp marker in the ladder. The inserted fragment of *gfpmut3a* is approximately 700-800 bp in size, so a sample of pBBR1MCS-2 with a successfully inserted *gfpmut3a* fragment should lie just in between the 5090 bp and 6108 bp markers of the ladder. Lane 5 of the gel in Figure 13, corresponding to isolated Culture #4, was approximately 1 kbp larger than pBBR1MCS-2 and also the plasmids isolated from the other cultures. Culture #4 was thus suspected to contain a derivative plasmid of pBBR1MCS-2 which has received the *gfpmut3a* gene. This plasmid was named pKN*lacgfp*.

To verify the presence of the *gfpmut3a* gene, the fluorescence signal of Culture #4 was measured and compared to two other *E. coli* cultures with GFP plasmids. As shown in Figure 14,

Culture #4 showed a comparable fluorescence signal to another strain of S17-1 λ-pir housing pAK*gfp*1, the source of *gfpmut3a*. Since both pAK*gfp*1 and a plasmid with successful insertion of *gfpmut3a* into pBBR1MCS-2 would operate under a *lac* promoter, the similarity in Culture #4 and S17-1 λ-pir / pAK*gfp*1's fluorescence signals was expected. Culture #4's fluorescence was also compared to the fluorescence of a culture of S17-1 λ-pir with a promoterless GFP plasmid, pAG408 (Suarez *et al*., 1997). S17-1 λ-pir / pAG408 was not expected to exhibit much fluorescence due to the lack of a promoter, and as expected Culture #4 and pAK*gfp*1 both produced substantially higher fluorescence signals than S17-1 λ-pir / pAG408. Culture #4's high fluorescence signal, coupled with the fact the culture was isolated from a kanamycin plate, gave confidence that Culture #4 was transformed by a derivative of pBBR1MCS-2 containing *gfpmut3a*. The GFP plasmid in Culture #4 was thus named pKN*lacgfp*, as the successfully constructed GFP and kanamycin resistant plasmid that expresses GFP under a *lac* promoter.

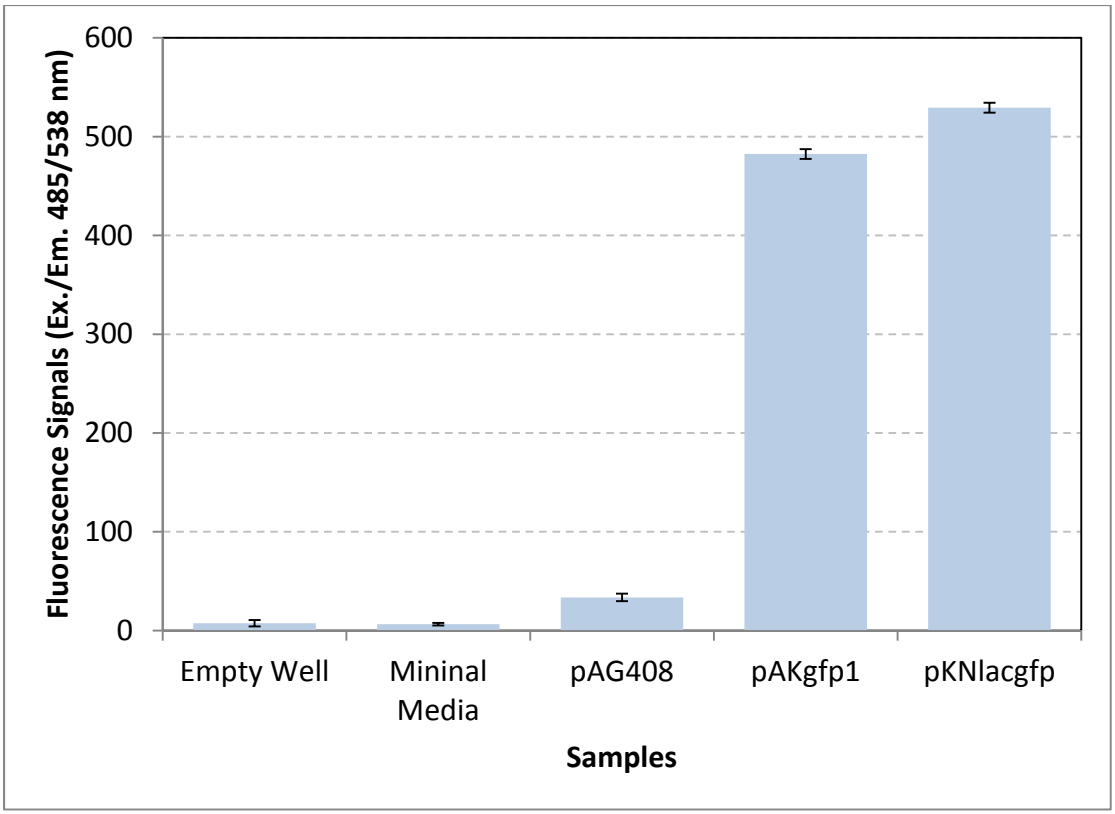

Figure 14: Fluorescence Signal of S17-1 λ-pir Cultures with Suspected pKN*lacgfp* vs. Other GFP Plasmids – Signals measured from wells which were: empty; minimal media; *E. coli* S17-1 λ-pir with promoterless GFP plasmid pAG408; *E. coli* S17-1 λ-pir with *lac* promoter GFP plasmid pAK*gfp*1; *E. coli* S17-1 λ-pir Culture #4 isolated from electroporation with suspected pKN*lacgfp*. Reported fluorescence signals are the average of 3 spectrophotometer readings on a single liquid batch culture of each strain. Liquid cultures were grown according to procedures outlined in 4.4.1. 1 mM of IPTG was added to each culture for the final 2 hours of growth. Cultures were pelleted and resuspended in minimal media for fluorescence measurements.

5.2. LABELING OF MC1071 WITH pKN*lacgfp*

To label MC1071 with pKN*lacgfp*, a culture of *E. coli* S17-1 λ-pir with pKN*lacgfp* (Culture #4 as described in Section 5.1. was conjugated with an active culture of MC1071. Conjugation was performed according to protocols outlined in Section 4.12. After the conjugation experiment, numerous colonies (too many to count) were observed on Min plates with (R)-2,4-DP and kanamycin. 50 of these cultures were restreaked on Min plates with (R)-2,4-DP and kanamycin. The fluorescence signals of the four cultures which grew most densely after restreaking were measured along with an MC1071 culture with no GFP plasmid. Since at

the time it had not been determined whether IPTG had an effect on the GFP expression of pKN*lacgfp* in *D. acidovorans*, 1 mM of IPTG was used to induce these cultures as described in Section 4.13. The fluorescence signals of all four potential GFP MC1071 cultures were substantially higher than the fluorescence measured from MC1071 without GFP. The four cultures have since been frozen and their GFP plasmids have been designated as pKN*lacgfp*-37, pKN*lacgfp*-38, pKN*lacgfp*-48, and pKN*lacgfp*-50.

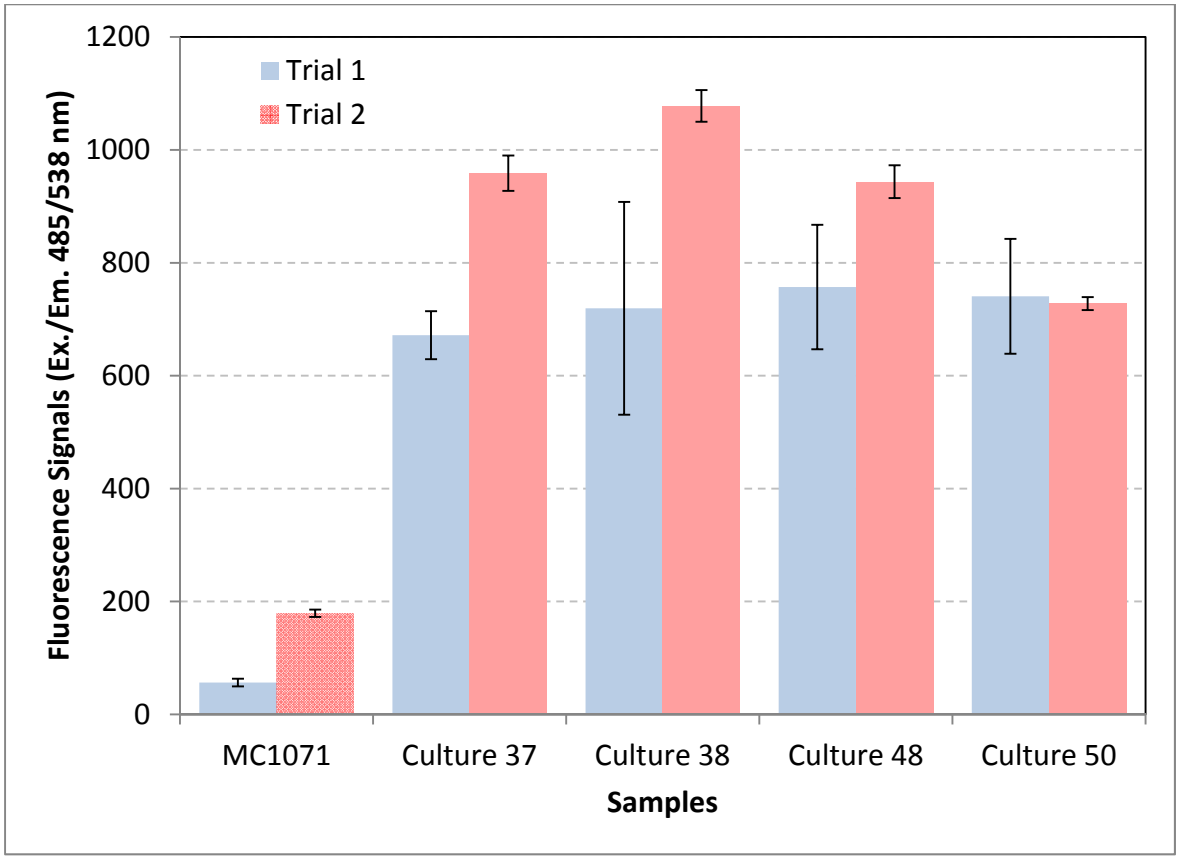

Figure 15: Fluorescence Signal of MC1071 Cultures Isolated after Transformation by Conjugation with pKN*lacgfp* Plasmids – Reported fluorescence signals are the average of 3 spectrophotometer readings on a single liquid batch culture of each strain. Liquid cultures were grown according to procedures in Section 4.4.2. 1 mM of IPTG was added to each culture for the final 2 hours of growth before spectrophotometric measurements were taken. Experiment was performed twice, with a fresh liquid culture grown each time.

When observed under microscope through DAPI filter, these four GFP transformed MC1071 cultures appear to be different from *E. coli*. They have also repeatedly shown successful degradation of (R)-2,4-DP in liquid culture. I therefore conclude that the cultures isolated from the conjugation experiment were in fact the recipient strain MC1071 and not the donor strain S17-1 λ-pir.

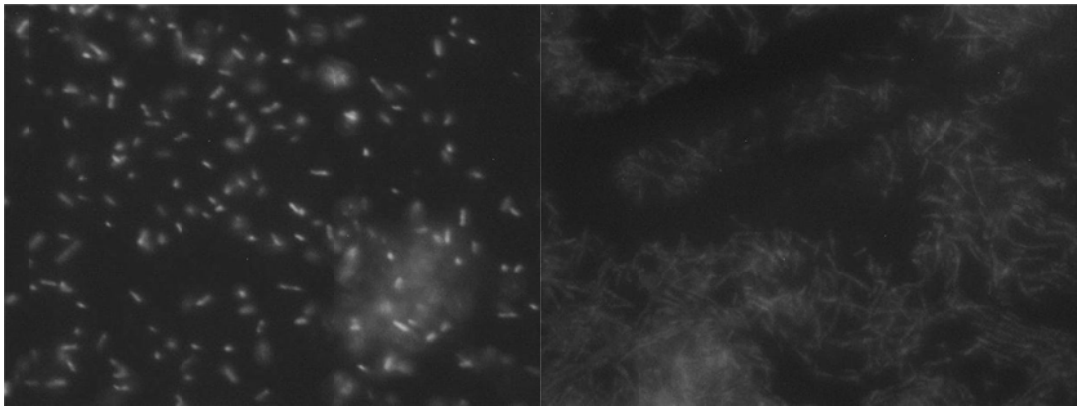

Figure 16: DAPI Microscopic Images of A) S17-1 λ-pir and B) MC1071 Through 100× Objective

The purpose of constructing pKN*lacgfp* and using it to label MC1071 was to produce a strain of MC1071 that would constitutively express GFP. The *lac* promoter is known to be regulated in certain gram-negative bacterial which produce *lac* repressors, such as *E. coli* (Hansen *et al.*, 1998). In *lac* repressor producing strains, lactose or an alternate inducer such as IPTG must be present for genetic expression to occur (Hansen *et al.*, 1998). It was unknown whether an inducer was required for genetic expression of *lac* promoter operated genes in *D. acidovorans*. Therefore, to test whether induction was required in pKN*lacgfp* labeled MC1071 for GFP expression, the fluorescence of pKN*lacgfp*-38 and pKN*lacgfp*-48 were measured with and without IPTG induction. Neither pKN*lacgfp*-38 nor pKN*lacgfp*-48 exhibited large difference

in fluorescence in the absence or presence of IPTG, thus showing that pKN*lacgfp* can constitutively express GFP in MC1071.

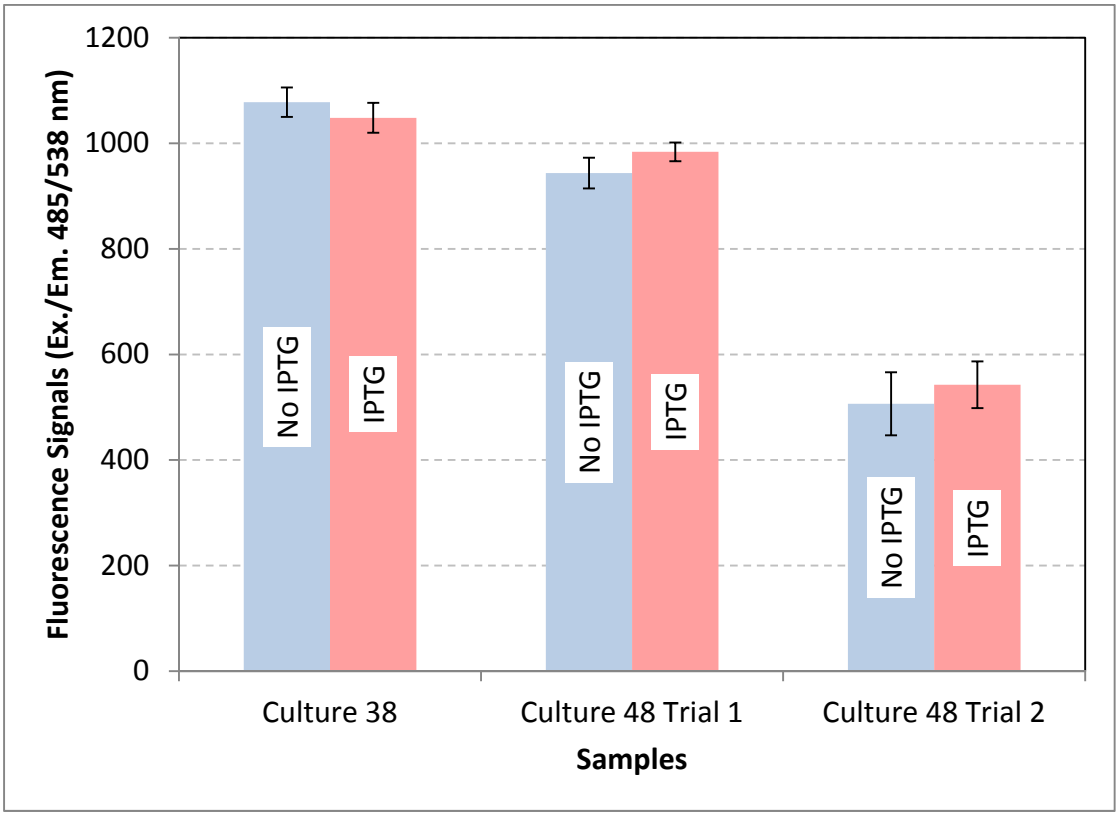

Figure 17: Effect of IPTG on the Fluorescence Signal of pKN*lacgfp*-38 and pKN*lacgfp*-48 in MC1071 – Reported fluorescence signals are the average of 3 spectrophotometer readings on a single liquid batch culture of each strain. Liquid cultures were grown according to procedures in Section 4.4.2. For cultures with IPTG, 1 mM of IPTG was added to each culture for the final 2 hours of growth before spectrophotometric measurements were taken.

5.3. CONSTRUCTION OF INDUCIBLE PLASMID pKN*tfdgfp*

Two ligation solutions were created to replace the *lac* promoter of pKN*lacgfp* with the *tfdC* promoter as described in Section 4.10.2. To screen for the plasmid with the correct promoter replacement, each ligation solution was electroporated into S17-1 λ-pir. After electroporation, the cultures were suspended in SOC media. One aliquot of 20 µL and two aliquots of 200 µL of each culture were spread on separate LB plates with kanamycin. Plate counts were performed

after overnight growth at 37°C. 63 and 65 cultures were counted from the plates spread with each electroporation mixture.

10 cultures transformed by each ligation solution were randomly selected and restreaked on fresh LB plates with kanamycin and grown overnight at 37°C. Plasmid extractions were performed on these 20 cultures. Plasmid extracts were digested with restriction enzyme *Xcm*I, which has one unique cutting site in pKN*lacgfp* and one unique cutting site in the section of *tfdC* fragment extracted from pUC/*tfdC* intended to be inserted into pKN*lacgfp*. Both undigested and digested plasmids from extractions were ran through 40 mL agarose gels by electrophoresis. The irradiated gels are shown in Figure 18 and Figure 19.

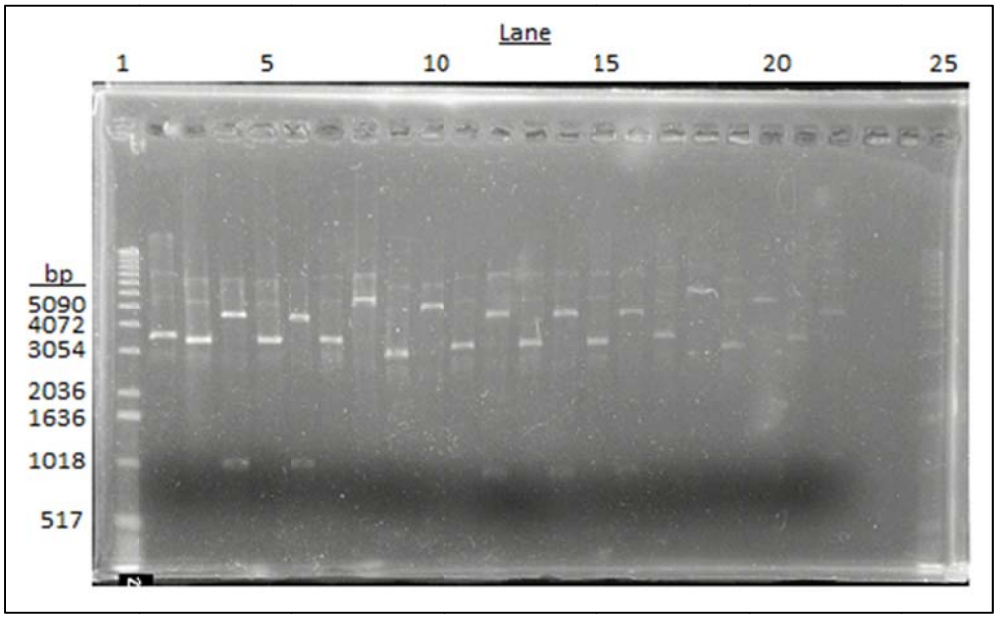

Figure 18: Screening Putative pKN*tfdgfp* Plasmids by Gel Electrophoresis (Cultures 1 – 10) – Lanes 1 and 25: Invitrogen standard 1 kB DNA Ladders; Lane 2: Undigested pKN*lacgfp*; Lanes 3-22: (Odd-numbered lanes undigested, Even-numbered lanes *Xcm*I digested) Plasmid extracts from S17-1 λ-pir cultures isolated from electrophoresis

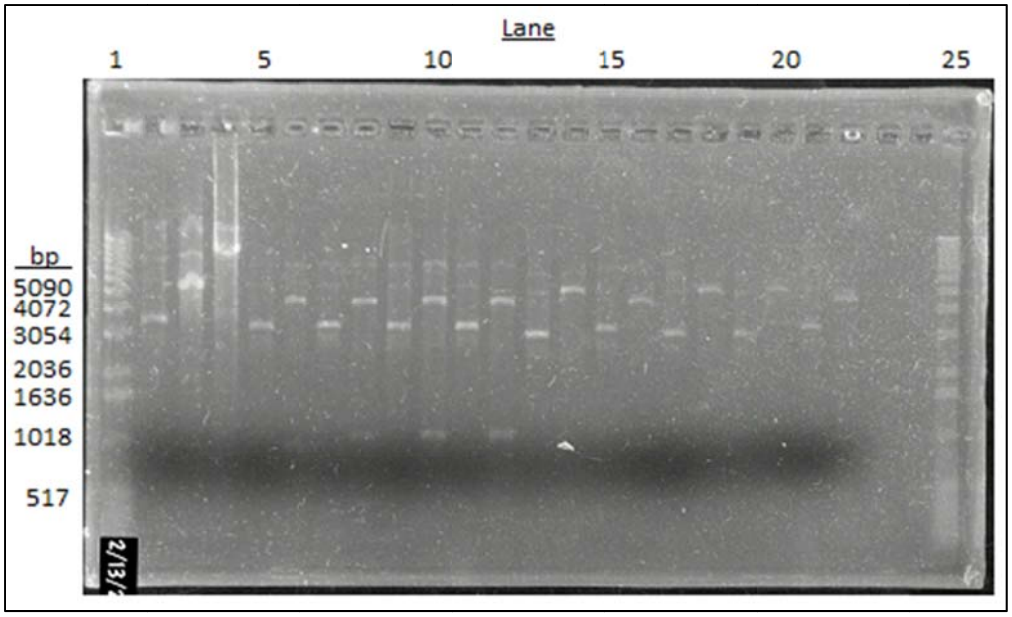

Figure 19: Screening Putative pKN*tfdgfp* Plasmids by Gel Electrophoresis (Cultures 11 – 20) – Lanes 1 and 25: Invitrogen standard 1 kB DNA Ladders; Lane 2: Undigested pKN*lacgfp*; Lanes 3-22: (Odd-numbered lanes undigested, Even-numbered lanes *Xcm*I digested) Plasmid extracts from S17-1 λ-pir cultures isolated from electrophoresis

Only plasmids where the *tfdC* fragment had successfully inserted into pKN*lacgfp* would have possessed two *Xcm*I digest sites, and therefore only these plasmids would have shown two fragments from an *Xcm*I digest: one fragment between 1 kbps to 2 kbps, and one fragment between 4 kbps and 5 kbps. From Figure 18 and Figure 19, we can see that S17-1 λ-pir cultures 1, 2, 5, 6, 7, 13, 14, 15 possessed plasmids which show very distinct bands within 517 to 1018 bps and 4072 to 5090 bps after *Xcm*I digest (Figure 18 lanes 4, 6, 12, 14, 16; Figure 19 lanes 8, 10, 12). These eight cultures were considered as possibly having been transformed by a ligation product of pKN*lacgfp* and a *tfdC* insert.

Cultures 2, 5, 13, and 15 were taken for further experiments to determine the presence of *gfpmut3a* by spectrophotometric measurements of their fluorescence signals. Additionally, the original GFP plasmid pBBR1MCS-2 expresses GFP under a *lac* promoter, which would require

IPTG induction for GFP expression in *E. coli* cultures. With successful promoter replacement the *gfpmut3a* would be operating with a *tfdC* promoter instead of a *lac* promoter, and thus GFP expression of a plasmid with correct promoter replacement in *E. coli* should not be affected by IPTG. Therefore, the fluorescence of Cultures 2, 5, 13, and 15 with and without the induction of IPTG were measured for comparison. All fluorescence measurements of Cultures 2, 5, 13, and 15 were compared to a culture of S17-1 λ-pir / pAG408 (Suarez *et al.*, 1997) containing a promoterless GFP plasmid which should show little fluorescence. Although IPTG should not affect the genetic expression of a plasmid without the *lac* promoter, the culture of S17-1 λ-pir / pAG408 was only measured with IPTG induction in case IPTG had an effect.

The fluorescence of cultures 2, 5, 13, and 15 were all higher than that of the culture with pAG408. Moreover, the fluorescence signals of these cultures were not affected by IPTG. The high fluorescence signals suggest that cultures 2, 5, 13, and 15 did in fact possess the *gfpmut3a* operating with a promoter. Because the expression of fluorescence in these *E. coli* cultures was not affected by IPTG, the *gfpmut3a* in these cultures is likely to be operating under a promoter other than the *lac* promoter. Coupled with the facts that these plasmids provided the cultures with kanamycin resistance, and that these plasmids were digested at two sites with the single *Xcm*I digest as is only predicted with the insertion of the *tfdC* promoter fragment into pKN*lacgfp*, plasmids of culture 2, 5, 13, and 15 were considered as successfully constructed GFP plasmids working under the *tfdC* promoter, and were designated pKN*tfdgfp*-2, pKN*tfdgfp*-5, pKN*tfdgfp*-13, and pKN*tfdgfp*-15 respectively.

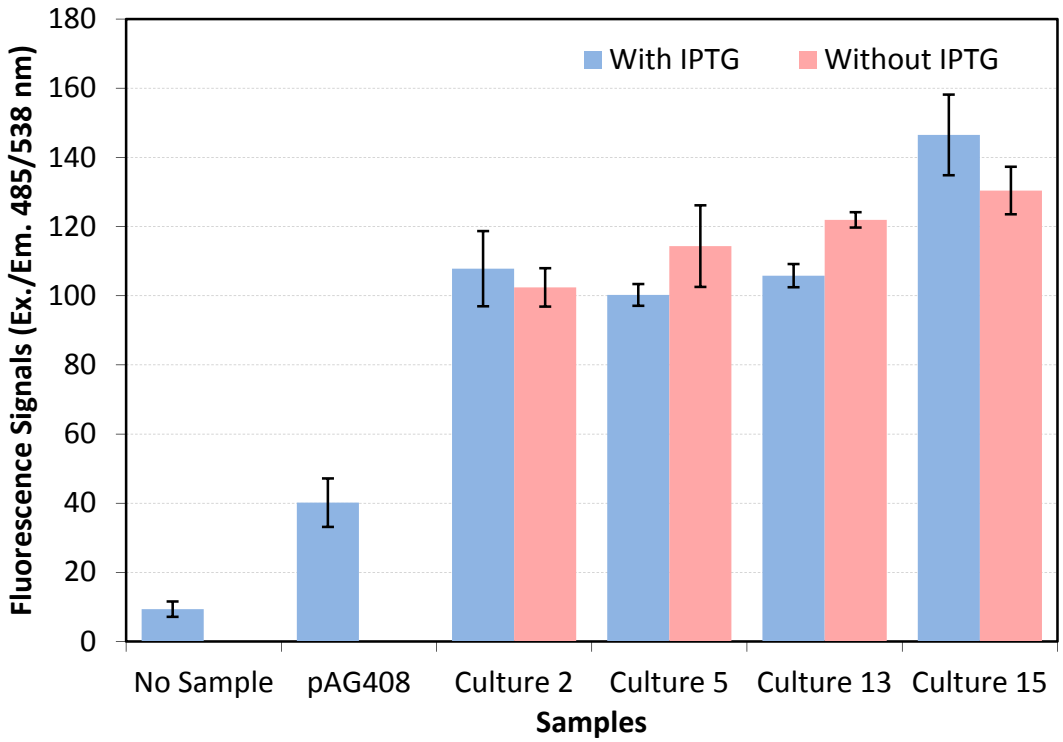

Figure 20: Fluorescence Signal of S17-1 λ-pir Cultures with Suspected pKN*tfdgfp* vs. Promoterless GFP Plasmid pAG408 – Signals measured from wells which were: Empty; *E. coli* S17-1 λ-pir with Promoterless GFP Plasmid pAG408; 4 cultures of *E. coli* S17-1 λ-pir isolated from electroporation with suspected pKN*tfdgfp*. Reported fluorescence signals are the average of 3 spectrophotometer readings on a single liquid batch culture of each strain. Liquid cultures were grown according to procedures outlined in 4.4.1. S17-1 λ-pir culture with pAG408 was not measured for its fluorescence signal without IPTG. For cultures with IPTG induction, 1 mM of IPTG was added to these cultures for the final 2 hours of growth. Cultures were pelleted and resuspended in minimal media for fluorescence measurements.

It should be noted that the fluorescence signals of *E. coli* with the pKN*tfdgfp* plasmids were much lower than the signal of the pKN*lacgfp* plasmids with IPTG induction (Figure 14). This is because the *tfdC* promoter of pKN*tfdgfp* requires the presence of a dichlorophenoxyalkanoic acid herbicide degradation products for induction of GFP expression (Vedler *et al.*, 2000). Since *E. coli* does not have the ability to degrade the herbicide, only basal levels of *tfdC* promoter (Kõiv *et al.*, 1996) and GFP expression were available from pKN*tfdgfp* in the tested *E. coli* cultures. The determination of whether pKN*tfdgfp* truly has its GFP expression inducible by herbicide

degradation will first require the transformation of *D. acidovorans* MC1071 by pKN*tfdgfp*, which is described in Section 5.4.

5.4. TRANSFORMATION OF MC1071 WITH pKN*tfdgfp*

To produce strains of *D. acidovorans* MC1071 which express GFP when induced by herbicide degradation, the plasmids pKN*tfdgfp*-2, pKN*tfdgfp*-5, pKN*tfdgfp*-13, and pKN*tfdgfp*-15 were conjugated from S17-1 λ-pir to MC1071 (isolation and identification of these plasmids are outlined in Section 5.3.). Conjugation was performed according to protocols outlined in Section 4.12. Transconjugants were isolated by growth on Min plates with (R)-2,4-DP to prevent S17-1 λ-pir growth and kanamycin to prevent growth of untransformed MC1071.

Confirmation of successfully transformed MC1071 was focused on the MC1071 culture conjugated with pKN*tfdgfp*-2. To determine whether MC1071 had truly been labeled by the GFP plasmid, the fluorescence signal of a MC1071/pKN*tfdgfp*-2 culture was compared to MC1071 with no GFP plasmid and MC1071/pKN*lacgfp*. MC1071/pKN*tfdgfp*-2 and MC1071/pKN*lacgfp* both provided higher fluorescence signals than MC1071 cultures no GFP plasmids, thus confirming the successful transformation of MC1071 with a GFP plasmid from conjugation. Furthermore, pKN*tfdgfp*-2 provided a much higher fluorescence to MC1071 than pKN*lacgfp*. Being native to *D. acidovorans*, the *tfdC* promoter was expected to provide more gene expression in MC1071 than the *lac* promoter which is native to *E. coli*, and therefore the higher fluorescence signal of pKN*tfdgfp*-2 than pKN*lacgfp* in MC1071 was expected.

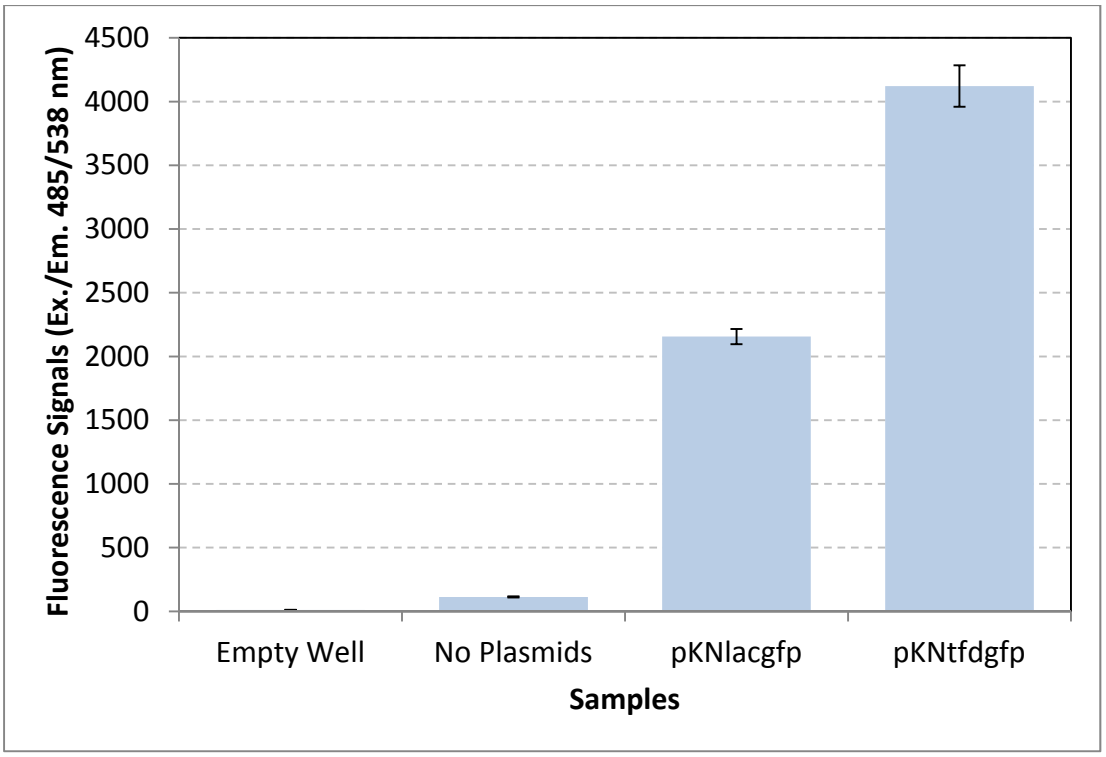

Figure 21: Fluorescence Signal of MC1071 Cultures with pKN*tfdgfp*-2 vs. other MC1071 Cultures – Reported fluorescence signals are the average of 3 spectrophotometer readings on a single liquid batch culture of each strain. Liquid cultures were grown according to procedures in Section 4.4.2.

To determine whether pKN*tfdgfp*-2's GFP expression was inducible by degradation of dichlorophenoxyalkanoic acid herbicides, the fluorescence signal of MC1071 cultures with pKN*tfdgfp*-2 grown on either the herbicide (R)-2,4-DP or the sugar D-mannitol were compared. Due to the large difference in cell density when MC1071 was grown on herbicide versus sugar, the fluorescence signals in Figure 22 were normalized by cell density. In Figure 22, the normalized fluorescence of MC1071 with pKN*tfdgfp*-2 when grown with (R)-2,4-DP was still much higher than MC1071 with no GFP plasmid. However, the normalized fluorescence of MC1071 with pKN*tfdgfp*-2 when grown with D-mannitol showed no difference from MC1071 with no plasmid. These results indicate that pKN*tfdgfp*-2 had its GFP expression induced when herbicide was present in culture growth. The strong fluorescence by pKN*tfdgfp*-2 in MC1071,

induced by the degradation of (R)-2,4-DP, shows the successful transformation of MC1071 by a herbicide-degradation induced GFP plasmid. Since the goal of these experiments was to produce just one strain of MC1071 with a herbicide-degradation induced GFP plasmid, pKN*tfdgfp*-2 has since been designated as pKN*tfdgfp*.

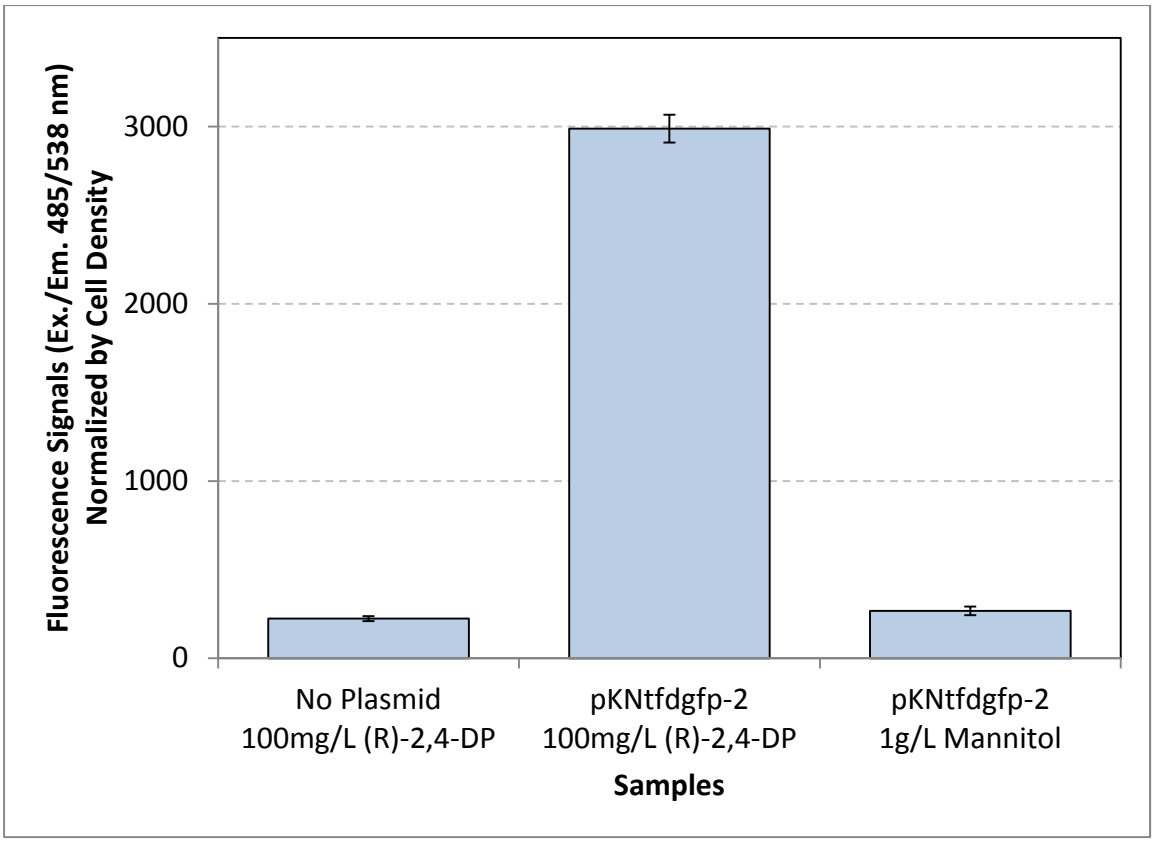

Figure 22: Normalized Fluorescence Signals of MC1071 Cultures with pKN*tfdgfp*-2 Grown on Herbicide or Sugar – Fluorescence signals were normalized by Cell Density. Cell density measurements were performed as the average of 3 absorbance readings at 600 nm. Fluorescence signals are the average of 3 spectrophotometer readings on a single liquid batch culture of each strain. Liquid cultures were grown according to procedures in Section 4.4.2.

CHAPTER 6: FUTURE WORK

6.1. STABILITY OF CONSTRUCTED PLASMIDS IN MC1071 IN THE ABSENCE OF SELECTIVE PRESSURE

Both GFP plasmids pKN*lacgfp* and pKN*tfdgfp* were constructed with kanamycin selectivity. MC1071 cultures containing either of the constructed GFP plasmids have thus far been maintained in media and plates with kanamycin to make sure that only cells with the plasmids can survive. Bacteria have a tendency to lose plasmids when selective pressures are not present (Imanaka and Aiba, 1981). It is therefore of interest whether the presence of kanamycin is required for MC1071 to maintain the two constructed GFP plasmids.

The stability of the plasmids is important for the long term study of competitive growth of GFP-labeled MC1071 and other herbicide degrading bacterial strains together in micromodels. In competitive experiments, GFP-labeled MC1071 will compete against other bacterial strains for substrates as described in section 6.4. If MC1071 can only maintain their GFP labeling in the presence of kanamycin, then the antibiotic will be required in micromodel experiments in order to maintain GFP labeling; in this case other strains will also need to be kanamycin resistant before they can survive in the micromodel. Conversely if MC1071 can retain GFP labeling in the absence of kanamycin, then other strains can grow together with the MC1071 in micromodels without kanamycin and the MC1071 strains will still maintain their GFP labeling.

To test for the stability of GFP plasmids in MC1071, batch cultures of GFP-labeled MC1071 will be grown with and without kanamycin. Periodically, samples will be taken from these cultures to be plated on regular LB plates and on LB plates with kanamycin. Plate counts will be performed on these samples. If the GFP plasmids are stable in MC1071 batch cultures

without kanamycin, then the number of cultures found on LB/kanamycin plates divided by the number of cultures found on regular LB plates and should remain constant over time. If, however, GFP plasmids are unstable in MC1071 batch cultures without kanamycin, then the number of cultures on LB plates with kanamycin divided by the number on regular LB plates will decrease over time. So far, this experiment has been performed once and the density of colony-forming units (CFU) were found as listed in Table 3.

Hours of Growth	CFU / mL on LB / Kanamycin Plates (A)	CFU / mL on LB Plates (B)	Ratio (A:B)
0	5.0×10^{10}	5.0×10^{10}	1
5	4.1×10^{10}	6.5×10^{10}	0.63

Table 3: CFU Density of MC1071/pKN*tfdgfp* Cultures Grown without Kanamycin – Cultures are grown in batch then samples are taken at 0 and 5 hours of growth and plated on A: LB/Km plates or B: LB plates. Experimental results inconclusive as some cultures unexpectedly changed appearance over time.

After 5 hours of growth in the absence of kanamycin, the ratio of kanamycin-resistant MC1071/pKNt*fdgfp* cultures in batch decreased. This indicates that kanamycin resistance may indeed have to be introduced into the other herbicide degrading bacterial strains intended for competition experiments in micromodels. Strangely the appearance and color of counted cultures changed several days after the experiment, leading to speculations of contamination either in the batch cultures or on plates; therefore these results were deemed inconclusive and repeats of the experiment will be carried out.

6.2. EFFECTS ON THE GROWTH RATE OF MC1071 BY CONSTRUCTED PLASMIDS

The expression of kanamycin resistance or GFP from pKN*lacgfp* and pKN*tfdgfp* requires expenditure of energy and substrates above basal levels required in culture maintenance. As a result, activity expression of either constructed plasmid might affect the growth rate of the cultures in which they are contained. Before studying the growth of GFP labeled MC1071

cultures in micromodels, we have to understand how the plasmids will affect the cultures' growth.

The growth rate of labeled MC1071 will be compared to unlabeled MC1071 in growth curve experiments. Both labeled and unlabeled MC1071 will be grown in minimal batch media with only herbicide as an electron donor. Samples will be taken periodically from each culture for optical density, fluorescence, and herbicide concentration measurements. Through the rate of increase in optical density, we can compare the growth rate of cultures. Fluorescence is expected to increase for labeled MC1071 cultures but not for unlabeled cultures. Herbicide concentration is expected to decrease for both cultures; whether there is a difference in the rate of herbicide degradation remains to be determined.

6.3. ADAPTATION OF GFP-LABELED MC1071 TO DEGRADE 2,4-D IN MICROMODELS

MC1071 adapts to degrading 2,4-D as a growth substrate in chemostats via post-translational modification of the enzyme RdpA (Leibeling, submitted 2011). The same adaptation has been studied in micromodel experiments (Yoon, submitted 2011), where MC1071 appeared to grow further away from (R)-2,4-DP sources and closer towards regions where both 2,4-D and O_2 were available as adaptation occurred. However, it was unclear which biomass in the micromodel experiment was actively degrading herbicide. The pKN*tfdgfp* tagged MC1071 strain was designed to only express GFP under active herbicide degradation. By repeating the adaptation experiment with the pKN*tfdgfp* tagged MC1071 strains, it is hoped that biomass actively degrading herbicide can be distinguished from biomass that is inactive by the difference in their fluorescence.

The expression of GFP by pKN*tfdgfp* tagged MC1071 must satisfy two criteria in order for the distinction of active and inactive biomass to be possible. First, tagged MC1071 must only express GFP when grown with a dichlorophenoxyalkanoic acid herbicide, either 2,4-D or 2,4-DP. The distinction can be made between tagged MC1071 grown with herbicide versus tagged MC1071 grown with sugar substrates. This experiment was performed as outlined in Figure 22 of Section 5.4. As expected, only tagged MC1071 grown with herbicide exhibited significant fluorescence signal, thus satisfying the criteria of tagged MC1071 only expressing GFP when induced with herbicide degradation.

Second, tagged MC1071 must lose its fluorescence some short period of time after it has expressed GFP; distinction of active and inactive biomass will be impossible if GFP which was produced by once actively herbicide-degrading biomass remains stable for too long after inactivity. To test for the stability of GFP in tagged MC1071, batch cultures of MC1071 can be grown with herbicide to the point of substrate exhaustion. The fluorescence signal and optical density of these MC1071 batch cultures will be continuously monitored after substrate exhaustion through spectroscopy. The cultures' fluorescence will be normalized by their optical densities. If GFP is unstable after biomass inactivity, then the normalized fluorescence signals of these MC1071 batch cultures should decrease over time.

6.4. COMPETITION OF GFP-LABELED MC1071 AND OTHER HERBICIDE DEGRADING BACTERIAL STRAINS IN MICROMODELS

GFP-labeled MC1071 can be visually distinguished from other non-fluorescent bacteria in micromodels when grown together. We can now study the competitive growth of MC1071 against other strains of dichlorophenoxyalkanoic acid herbicides in micromodels to further

understand the spatiotemporal effects of competitive microbial growth. Candidate strains for competition against MC1071 include *D. acidovorans* MC1 $RdpA^{+}/SdpA^{+}$, MC1010 $RdpA^{-}/SdpA^{+}$, MC1100 $RdpA^{-}/SdpA^{-}$, and *Ralstonia eutropha* JMP 134 $TfdA^{+}$. Each of these strains can compete for different herbicide substrates with MC1071 and can provide different amounts of stress to MC1071 growth, which in turn may lead to different growth patterns of MC1071 in the micromodel.

CHAPTER 7: CONCLUSION

In these experiments, two new GFP plasmids, pKN*lacgfp* and pKN*tfdgfp*, were successfully constructed. These two plasmids were transferred into *E. coli* S17-1 λ-pir and *D. acidovorans* MC1071. Both pKN*lacgfp* and pKN*tfdgfp* provide S17-1 λ-pir and MC1071 with resistance to the antibiotic kanamycin. pKN*lacgfp*'s GFP expression in S17-1 λ-pir is greatly increased in the presence of IPTG, but its GFP expression in MC1071 is not affected by IPTG. pKN*tfdgfp*'s GFP expression is not affected by IPTG in either S17-1 λ-pir or MC1071. pKN*tfdgfp*'s GFP expression in MC1071 is induced by a dichlorophenoxyalkanoic acid herbicide (R)-2,4-DP, but not on an alternate carbon source of D-mannitol.

The successful transformation of MC1071 by pKN*lacgfp* and pKN*tfdgfp* is one step in the long-term study of bacterial adaptation and competition in micromodels. Next, the interactions between MC1071 and the GFP plasmids will be studied in detail. Specifically, we will determine whether the GFP plasmids will affect MC1071's growth rate or density, whether kanamycin selectivity is required to maintain plasmid stability in MC1071, and whether GFP expression is stable in active and inactive MC1071. After understanding the interactions of the GFP plasmids with MC1071, we will then employ the GFP-tagged MC1071 strains in micromodel experiments. The pKN*lacgfp*-tagged MC1071 is designed for visual distinction of MC1071 from other bacterial strains in micromodels; this will allow for studies of bacterial competition in a spatially structured environment. The pKN*tfdgfp*-tagged MC1071 will allow distinction of active and inactive MC1071 biomass in micromodels; this is for studies of bacterial adaptation in an environment limited by physical factors such as substrate mass transfer, as opposed to previous studies of bacteria adaptation in well mixed batch cultures. At the completion of these adaptation

and competition micromodel experiments, we will have a greater understanding of how spatial patterns can affect the behavior of biomass in simulated soil systems.

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