IDENTIFICATION OF GENES INVOLVED IN THE PRODUCTION OF

A NOVEL ANTIFUNGAL AGENT (7, 10, 12-TRIHYDROXY-8(*E*)-

OCTADECENOIC ACID) IN *PSEUDOMONAS AERUGINOSA*

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Jessica Cofield

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IDENTIFICATION OF GENES INVOLVED IN THE PRODUCTION OF A NOVEL ANTIFUNGAL AGENT (7, 10, 12-TRIHYDROXY-8(*E*)-OCTADECENOIC ACID) IN *PSEUDOMONAS AERUGINOSA*

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VITA

Jessica Alys Cofield, daughter of Bill and Sally Cofield was born on 28 April 1983 in Cullman, Alabama. She graduated from Oneonta High School, Oneonta, Alabama, in 2001. Miss Cofield received a Bachelor of Science degree in Microbiology from Auburn University, Auburn, Alabama, in 2005. Upon completion of her B.S., Miss Cofield entered the Graduate School, Auburn University, Alabama, in May 2005, but it was not until October 2005 that she began work towards a Master of Science degree in Microbiology.

THESIS ABSTRACT

IDENTIFICATION OF GENES INVOLVED IN THE PRODUCTION OF A NOVEL ANTIFUNGAL AGENT (7, 10, 12-TRIHYDROXY-8(*E*)-OCTADECENOIC ACID) IN *PSEUDOMONAS AERUGINOSA*

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The gram-negative bacterium *Pseudomonas aeruginosa* catalyzes the conversion of ricinoleic acid into a novel trihydroxy fatty acid, 7, 10, 12-trihydroxy-8(*E*)-octadecenoic acid (TOD), that has a potent antifungal activity against important crop pathogens, including *Magnaporthe grisea* the causative agent of rice blast disease. Natural crop-protecting agents such as TOD offer several advantages over synthetic agents, including improved ecological compatibility and environmental safety. Unfortunately, because many naturally occurring crop-protecting agents are produced only in trace amounts, it has been difficult to isolate large enough quantities of these

antimicrobial agents to be economically feasible. Thus, a bacterium such as *P. aeruginosa* that is genetically amenable and produces an antifungal agent is ideal for genetic manipulation to achieve improved TOD production. The long-term goal of this research is to develop efficient processes for improving production of TOD from *P. aeruginosa* to mass-produce an economically competitive and environmentally friendly biological crop protection agent. As an initial step towards this goal, a genetic study was undertaken to identify the genes that are required for production of TOD in *P. aeruginosa*. In order to facilitate the genetic screen, a bioassay was developed to assess TOD's antifungal activity against *M. grisea* in 96-well microtiter plates using either pure TOD or *P. aeruginosa* culture supernatants. In order to identify the genes involved in the bioconversion, a transposon mutagenesis was performed and a library of ~15,000 Tn*5*-B21 insertion mutants of the *P. aeruginosa* strain NRRL B-23260 (an environmental isolate) was constructed. From the genetic screen, 28 transposon insertion mutants that were defective for TOD production were identified. Molecular studies identified eleven mutants that appeared to have single transposon insertions. Genetic mapping of these eleven mutants was conducted to identify the genes that had been interrupted by transposon insertion. From this analysis, eight genes were identified including genes encoding alkylated DNA repair proteins, ferrous iron transport proteins, pseudouridylate synthase, a hypothetical protein/magnesium transport protein, a transport protein of ABC transport, and XcpP of the general secretory pathway. Three of the mutants have been successfully complemented with the wild-type genes to demonstrate their involvement in the microbial conversion of ricinoleic acid to TOD.

ACKNOWLEDGEMENTS

I would, first, like to thank Dr. Sang-Jin Suh for taking me on as a graduate student and for his scientific guidance. I would also like to thank Dr. Silo-Suh, Dr. Barbaree, Dr. Brown, and Dr. Lawrence for their guidance throughout this process and for agreeing to be on my committee. I want to show gratitude to Taylor Boozer Hatchett for her contribution to this research project. Dr. Tsung-Min Kuo of Microbial Genomics and Bioprocessing Group (USDA, ARS, NACUR, Peoria, IL) provided me with TOD and methods for TOD production used in this project, and so I emphatically say "Thank you!" to him. Regrettably, Dr. Kuo passed away about two years ago. I wish to say, "Thank you!" to all of the past and present members of the Suh and Silo-Suh labs for their friendship and kindness through the years. I want to thank John McInroy for his assistance in preparation and analysis of samples for GC. And to Yi Liu, "Thank you!" for your indispensible assistance with cloning. To my sister, Jennifer, I thank you for encouraging me to be more than I am, and I want to thank my brother, Joseph, for always being able to make me laugh and for just being there when I needed to talk to someone. Of course, I cannot forget to thank my parents who are my everything. They have always been there for me, always encouraged me to go the distance, and for being my rock. Last, but certainly not least, I wish to thank God for all his blessings and never ending love and support. WAR EAGLE!!

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CHAPTER 1

LITERATURE REVIEW

1.1. 7, 10, 12 - trihydroxy- 8(*E*)-octadecenoic acid and anti-microbial activity

Fatty acids are carboxylic acids with a hydrocarbon on one end and a carboxyl group at the opposite end. They vary in length (10 – 22 carbons), are predominantly unbranched, and can be saturated or unsaturated. They are found in plant, animal, and microbial cells as energy storage units, cell membrane precursors (i.e. lipids), or hormone precursors (chemicalland21.com, 2008). Synthetic long-chain fatty acids can be derived from these naturally occurring fatty acids by a conversion process within plant, animal or microbial cells (Lie Ken Jie, *et al*., 1998). Exogenous long-chain fatty acids can be transported across the cell membrane of most cells for use as metabolic energy or for conversion (DiRusso and Black, 1999). Specific membrane proteins are involved in transport, which supports the hypothesis of fatty acid entry being highly specific and regulated (DiRusso and Black, 1999). Hydroxylated fatty acids from plants play roles in defense against microbial pathogens, stimulation of gene expression in plant defense, and regulation of plant cell death (Prost, *et al.*, 2005). A naturally occurring fatty acid in corn oil, oleic acid, can be hydrolyzed to form a monohydroxy-unsaturated fatty acid, ricinoleic acid, within a plant or bacterial cell (Eckey and Miller, 1954; El-Sharkawy, *et*

al., 1992). Ricinoleic acid is a C_{18} fatty acid that is found in castor beans, *Ricinus communis*, from the family Euphoribiaceae. Castor beans store ricinoleic acid as the major storage fatty acid within spherosomes of growing gymnosperms. Ricinoleic acid can be found during all stages of germination, but the amount drastically increases in the later stages of development until it reaches up to 80% of the triacylglycerol in gymnosperms (Donaldson, 1977; El-Sharkawy, *et al*., 1992; James, *et al*., 1965; Piazza and Farrell, 1991; Vick and Beevers, 1978). Thus, the major commercial source for ricinoleic acid, which is used in textile finishing, coating, ink, and making soaps, comes from castor bean gymnosperms (Bafor, *et al*., 1991; chemicalland21.com, 2008). Monohydroxy fatty acids, such as ricinoleic acid, can serve as precursors to dihydroxy and trihydroxy fatty acids. Trihydroxy fatty acids have been isolated from both plants and microbial organisms (Hou 1998; Hou and Forman, 2000; Kuo and Nakamura, 2004; Lie Ken Jie, *et al*., 1998). In 2001, Kuo and Hou demonstrated that ricinoleic acid serves as a precursor to a trihydroxy-polyunsaturated fatty acid, 7, 10, 12 - trihydroxy- 8(*E*)-octadecenoic acid (TOD) (Kuo and Hou, 2001). Trihydroxy fatty acids, including TOD, have been shown to possess anti-microbial activities (Lie Ken Jie, *et al*., 1998). TOD is effective against plant fungal pathogens that cause peach blossom blight, potato late blight, rice blast disease, and rice sheath blight. TOD also has activity against some insects, including corn planthopper, green peach aphid, two-spotted spider mite, and some flies' eggs and larva. However, TOD was most effective against *Magnaporthe grisea*, the causative agent of rice blast disease (Hou and Forman, 2000; Kato, *et al*., 1985; Kuo and Hou, 2001; Kuo, *et al*., 2001).

Plants mount a local and systemic defense against fungal germ tube penetration and propagation in the plant cells by generation of intracellular signals. Trihydroxy fatty acids, such as TOD, inhibit the germination and elongation of the fungal conidial germ tubes (Kato, *et al*., 1985; Vick and Beevers, 1978). Although the exact mechanism of TOD's activity has not yet been elucidated, Avis and Belanger (2001) demonstrated that fatty acids can alter the membrane fluidity of a cell by integrating into the lipid bilayer or inclusion in fatty acyl-chains of phospholipids. The *cis* double bond increases the activity of fatty acids, while addition of a second double bond increases its toxicity further (Kabara, *et al*., 1972). In addition, fatty acids with double bonds bind and alter membrane sterols to destabilize the integrity of the membrane and its function (Avis and Belanger, 2001). The hydroxyl group(s) provides fatty acids with a increased viscosity and reactivity. The location of the hydroxyl groups in a fatty acid may influence the specificity of the fatty acid against a particular cell type including those of plant pathogenic fungi (Hou and Forman, 2000).

Microorganisms are capable of producing fatty acids that have antimicrobial properties. Hou and Forman (2000) tested three types of hydroxy fatty acids for anti-fungal activity against common plant pathogenic fungi and determined that the specificity of trihydroxy fatty acids may depend on the location of the hydroxyl groups on the fatty acid molecule. A survey of TOD was conducted to test its biological activity against plant pathogenic fungi and bacteria, plant growth promoting bacteria, and nematodes. Of the 23 plant pathogenic fungi screened, only *M. grisea* was completely inhibited by TOD

at 0.01 ppm (Hatchett, *et al*., 2009). TOD was also effective against many soil bacteria, inhibiting the growth of 15 out of 17 bacteria tested. However, TOD was ineffective against nematodes *Rotylenchulus reniformis* and *Meloidogyne incognita* (Hatchett, *et al*., 2009). These data demonstrate a highly specific activity of TOD against plant pathogenic fungi.

1.2. TOD production by *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is a Gram negative, motile, rod-shaped bacterium in the class Gamma Proteobacteria. It is almost ubiquitous in the environment but is more commonly found in soil and water habitats. This bacterium is capable of utilizing a vast range of organic compounds for nutrients and this metabolic versatility facilitates *P. aeruginosa* to survive and thrive in a multitude of environments (Todar, 2008). *P. aeruginosa* is also an opportunistic bacterial pathogen that mainly affects burn victims, cancer, cystic fibrosis and AIDS patients. *P. aeruginosa* possesses a genome of 6.3 million base pairs with approximately 5570 possible open reading frames (Stover, *et al*., 2000). The large genome size and estimated 500 transcriptional regulators enhance the ability of this bacterium to sense, respond, and adapt to environmental alterations. *P. aeruginosa* is very effective in the metabolism of organic substances because its genome encodes a vast variety of transporters for mono-, di-, and tri-carboxylates (Stover, *et al*., 2000).

P. aeruginosa produces a number of value-added compounds including biosurfactants (rhamnolipids) and biodegradable plastic (polyhydroxyalkanoic acids). In 2001, Kuo and colleagues isolated strains of *P. aeruginosa* from a wastewater stream on a pig farm in Illinois that converted monohydroxy-unsaturated fatty acid (ricinoleic acid) into a trihydroxy-polyunsaturated fatty acid, 7, 10, 12 - trihydroxy- 8(*E*)-octadecenoic acid (TOD) (Kuo and Hou, 2001; Kuo, *et al*., 2001). Subsequently, it has been determined that most *P. aeruginosa* isolates can catalyze this conversion. The conversion of ricinoleic acid to TOD by *P. aeruginosa* is believed to be accomplished via two hydroxylation steps. The first step includes a double bond migration from the C 9-10 position to the C 8-9 position and the addition of another hydroxyl group on ricinoleic acid to form 10, 12 – dihydroxy-octadecenoic acid (DOD). Subsequent hydroxylation at C-7 of DOD results in the production of 7, 10, 12 –trihydroxy-octadecenoic acid (TOD) (Kuo and Nakamura, 2004). In 2004, Kuo and colleagues demonstrated that the enzymes necessary for the conversion process appeared to be induced by the addition of exogenous ricinoleic acid (Kuo and Nakamura, 2004). During the conversion steps, ricinoleic acid is almost completely converted to DOD and/or TOD by *P. aeruginosa*.

1.3. Rice blast disease and the impact of *Magnaporthe grisea*

Various microorganisms cause plant disease but fungi comprise some of the most important pathogens. Defense mechanisms in plants include a thick, complex cell wall, genetic resistance, signaling pathways, and oxylipins (defense response particles) (Sexton and Howlett, 2006). However, even with such a formidable defense, plants are

susceptible to various pathogens. Fungi invade plant cells by taking advantage of natural openings, intentionally causing wounds with toxins, mechanical force, or producing invasive hyphae to penetrate cell walls (Park, *et al*., 2005; Sexton and Howlett, 2006). *Magnaporthe grisea*, an ascomycete, is the causative agent of rice-blast disease and poses the greatest threat to rice, which is one of the most important agricultural crops for 6 billion people worldwide (Zeigler, *et al*., 1994). It is estimated that each year the rice yields lost to rice blast would feed 60 million people (Correll, *et al*., 2000; Smith, 1999; Zeigler, et al., 1994). In continuing attempts to control *M. grisea* and its effects, farmers have been using synthetic fungicides as preventative and therapeutic control measures. Unfortunately, overuse of fungicides has led to environmental pollution and emergence of resistant pathogens. Rice blast has also been controlled by developing resistant cultivars of rice but these are temporary measures due to the emergence of new and more virulent *M. grisea* strains (Chao and Ellingboe, 1996; Park, *et al*., 2005). The *M. grisea* population is not diverse in the United States. There are eight known genetic variations with only four that are commonly seen in infected rice plants (Chao and Ellingboe, 1996; Correll, *et al*., 2000). The ability to penetrate the cuticle of plant cells is one of the reasons pathogenic fungi are successful in causing infections. During the infection, *M. grisea* fungal hypha contacts the surface of the rice plant and the conidium begins to germinate and forms a germ tube. The end of the germ tube differentiates into a dome-shaped, melanized appressorium, a specialized cell that attaches strongly to the plant surface. A rapid increase in turgor in the appressorium due to accumulation of intracellular glycerol results in rupturing of the plant cuticle (Balhadere and Talbot,

2001). Following rupture, the end of the appressorium differentiates into a "penetration peg" that breaks through the plant cuticle and epidermal cell wall. The "penetration peg" undergoes rapid cell wall synthesis due to the continued turgor pressure that the appressorium exerts on the elongating hyphal cell. It becomes a bulb-like infective hypha with branched structures that grow between and within plant cells (Balhadere and Talbot, 2001; Smith, 1999). The branched structures then continue to grow and kill the host plant cells. An anamorph (asexual reproductive stage) of *M. grisea*, *Pyricularia grisea*, is the causative agent of gray leaf spot on perennial rye grass. The first reported case of gray leaf spot occurred in Pennsylvania in 1992 (Farman, 2001). *P. grisea* has become the most destructive and devastating of turf grass diseases in the United States. Initially, scientists believed *M. grisea* and *P. grisea* could only infect rice. However, the possibility of cross-infection between species was confirmed in a 1992 Pennsylvania case. It was later determined that gray leaf spot was not caused by an "opportunistic" infection by *P. grisea*, but by a population of *P. grisea* that is specific to perennial rye grasses (Farman, 2001).

1.4. Hypothesized enzymes involved in TOD conversion

Although the precise mechanism for conversion of ricinoleic acid to TOD by *P. aeruginosa* is unknown, the conversion process is hypothesized to be catalyzed by enzymes, such as lipoxygenase and monooxygenase. *P. aeruginosa* contains one probable lipoxygenase (*PA1169*) whose function has not yet been characterized. The bacterium also contains at least 11 and as many as 54 genes that may encode for a

monooxygenase. Lipoxygenase is generally associated with the "abstraction" of hydrogen and insertion of molecular oxygen at carbon-carbon double bond sites (hydroxylation) in fatty acids (Vance *et al*., 2004; Vick and Zimmerman, 1987). They are characterized as non-heme iron containing dioxygenases that catalyze the oxygenation of polyunsaturated fatty acids (Vidal-Mas, *et al*., 2005). This enzyme has been studied in plants, animals, and bacteria (Porta and Rocha-Sosa, 2001; Vidal-Mas, *et al*., 2005). In plants, there are four enzyme systems actively involved in fatty acid modification; including the lipoxygenase pathway (Vick and Zimmerman, 1987). Rice has been reported to possess three different isoenzymes of lipoxygenase, each with different product specificity (Vick and Zimmerman, 1987). Fatty acids are substrates for lipoxygenase and oxygen is usually incorporated into the C-6 or C-10 positions (Vick and Zimmerman, 1987). The lipoxygenase reaction catalyzes a hydroperoxide product which results when a *cis* double bond is attacked by oxygen and binds to a neighboring *cis* double bond, which then migrates to assume a *trans* configuration (Steczko, *et al*., 1992; Vick and Zimmerman, 1987). All lipoxygenases contain one atom of iron, which is bound by conserved histidine residues (a common ligand of iron in many proteins) (Porta and Rocha-Sosa, 2001; Steczko, *et al*., 1992). The iron in lipoxygenase is essential for catalysis and cycles between iron (II) and iron (III) (Vial-Mas, *et al*., 2005; Vick and Zimmerman, 1987). In *P. aeruginosa*, it is assumed that lipoxygenase is secreted from the cytoplasm and becomes active in the periplasmic space and supernatant (Vance, *et al*., 2004; Vidal-Mas, *et al*., 2005). It is assumed that the secretion of lipoxygenase is dependent on its enzymatic activity (Steczko, *et al*., 1992; Vance, *et al*., 2004). Vance, *et*

al. (2004) demonstrated that lipoxygenase was secreted out of the cell by constructing an *xcp* deletion, involved in type II secretion. Furthermore, an 84% reduction in lipoxygenase activity was observed in supernatants of the Xcp mutant. In this study, the investigators demonstrated that although the lipoxygenase was localized to the periplasm, a significant amount of the enzyme was also secreted to the extracellular milieu (Vance, *et al*., 2004).

Monooxygenase adds molecular oxygen to a substrate coupled with the reduction of a second oxygen atom to water (Prigge, *et al*., 1997). In the reaction hydrogen is removed from the substrate followed by oxygen being bound (hydroxylation) to the substrate (Gibson, *et al*., 1995). Monooxygenase reactions have been compared to those catalyzed by cytochrome P-450 (Gibson, *et al*., 1995). Activation requires the enzyme to be in a ternary or reduced conformation in order to produce hydroxylated products and water (Prigge, *et al*., 1997). Monooxygenase contains di-iron clusters at the active site and can be classified as a mixed function oxygenase where the majority catalyzes hydroxylation of aromatic rings (Gibson, *et al*., 1995). However, the substrate range is not limited to aliphatic compounds (Britton and Markovetz, 1977). The exact interaction of monooxygenase with fatty acids is unknown but the hydroxylation of ricinoleic acid to DOD or DOD to TOD is possible.

1.5. Summary

M. grisea is an important pathogen worldwide of rice and of turf grass in United States. The novel 7, 10, 12 - trihydroxy- 8(*E*)-octadecenoic acid (TOD) has high specificity and antifungal activity against *M. grisea* that can be utilized to combat the diseases caused by this fungus. The bacterium *P. aeruginosa* catalyzes conversion of ricinoleic acid to TOD. The conversion process is hypothesized to be catalyzed by lipoxygenase and/ or monooxygenase classes of enzymes. The long-term goal of this study is to genetically engineer *P. aeruginosa* for enhanced TOD production. As an initial step towards the long-term goal, the purpose of my study was to identify *P. aeruginosa* genes involved in TOD conversion.

CHAPTER 2

IDENTIFICATION OF Tn*5*-B21 INSERTION MUTANTS OF *PSEUDOMONAS AERUGINOSA* DEFICIENT IN TOD PRODUCTION VIA A HIGH THROUGHPUT ANTIFUNGAL ASSAY

2.1. Introduction

Rice is a semi-aquatic plant that is cultivated in five ecosystems, such as in deepwater and in tidal wetlands. It is a substantial crop and food source in Southeast Asia for about 100 million people (Kende, *et al*., 1998). Modern cultivars of rice can produce up to 60 million tons of rice per hectacre (Kende, *et al*., 1998). *Pseudomonas aeruginosa*, a gram-negative bacterium, catalyzes the conversion of ricinoleic acid into a novel trihydroxy fatty acid, 7, 10, 12-trihydroxy-8(*E*)-octadecenoic acid (TOD). TOD is a potent antifungal agent against important crop pathogens, more specifically against *Magnaporthe grisea* which causes rice blast disease (Kuo and Nakamura, 2004). Chemical treatment of rice blast is accomplished through the use of fungicides and is most effective when applied preventatively. Natural crop-protecting agents such as TOD offer several advantages over synthetic agents, including improved ecological compatibility and environmental safety. Plants produce several hydroxylated fatty acids that possess antifungal activities (Kato, *et al*., 1984; Kato, *et al*., 1985; Kato, *et al*., 1986;

Masui, *et al*., 1989). Mixed hydroxy fatty acids have been isolated from rice plants that showed anti-fungal activity (Hou, 1996). Their structures were identified as 9*S*, 12*S*, 13*S*-trihydroxy-10-octadecenoic acid and 9*S*, 12*S*, 13*S*-trihydroxy-10, 15-octadecadienoic acid, which showed anti-fungal activity against the causative agent of black rot, *Ceratocystis fimbriata* (Hou, 1996; Hou and Forman, 2000). (Figure 2.1) Unfortunately, these compounds are produced only in minute quantities, which makes it difficult to isolate large amounts in a cost effective manner (Hou, 1998; Hou, *et al*., 1997).

The proposed conversion of ricinoleic acid, derived from castor beans, to TOD involves the hydroxylation of C-7 and C-10 which results in the rearrangement of the double bond from C 9–10 (*cis*) to C 8–9 (*trans*) as illustrated in Figure 2.2. Further hydroxylation of a secondary dihydroxy fatty acid, designated DOD, results in TOD (Kuo and Hou, 2001). Rice blast is considered to be the most common and most devastating disease of cultivated rice and is characterized by visible lesions on the leaves of rice plants three to five days post-infection (Dong, *et al*., 2007; Odenbach, *et al*., 2007). In addition to rice-blast, the asexual stage of *M. grisea*, designated as *Pyricularia grisea*, causes gray leaf spot disease on turf grasses throughout the United States. In high humidity, the lesions produced by *P. grisea* turn a characteristic gray and result in death of the grass blade (Dong, *et al*., 2007). A survey of TOD was conducted to test its biological activity against plant pathogenic fungi and bacteria, plant growth promoting bacteria, and nematodes. As indicated in Hatchett, *et al*. (2009), TOD completely

inhibited *M. grisea* and soil bacteria but was ineffective against other pathogenic fungi and nematodes (Hatchett, *et al*., 2009).

The major goal of this research is to genetically engineer *P. aeruginosa* to maximize TOD production and to use it as an economically competitive and environmentally friendly biological crop protection agent. As an initial step, *P. aeruginosa* mutants defective for TOD production were generated via transposon mutagenesis then identified. To facilitate genetic screening of a large number of potential mutants, a high throughput antifungal assay was developed. Utilizing this assay, approximately 15,000 mutants were screened for production of TOD by assessing the inhibitory activity on *M. grisea* growth. The high throughput bioassay was developed in collaboration with Dr. Katheryn Lawrence and Taylor B. Hatchett of the Plant Pathology Department at Auburn University.

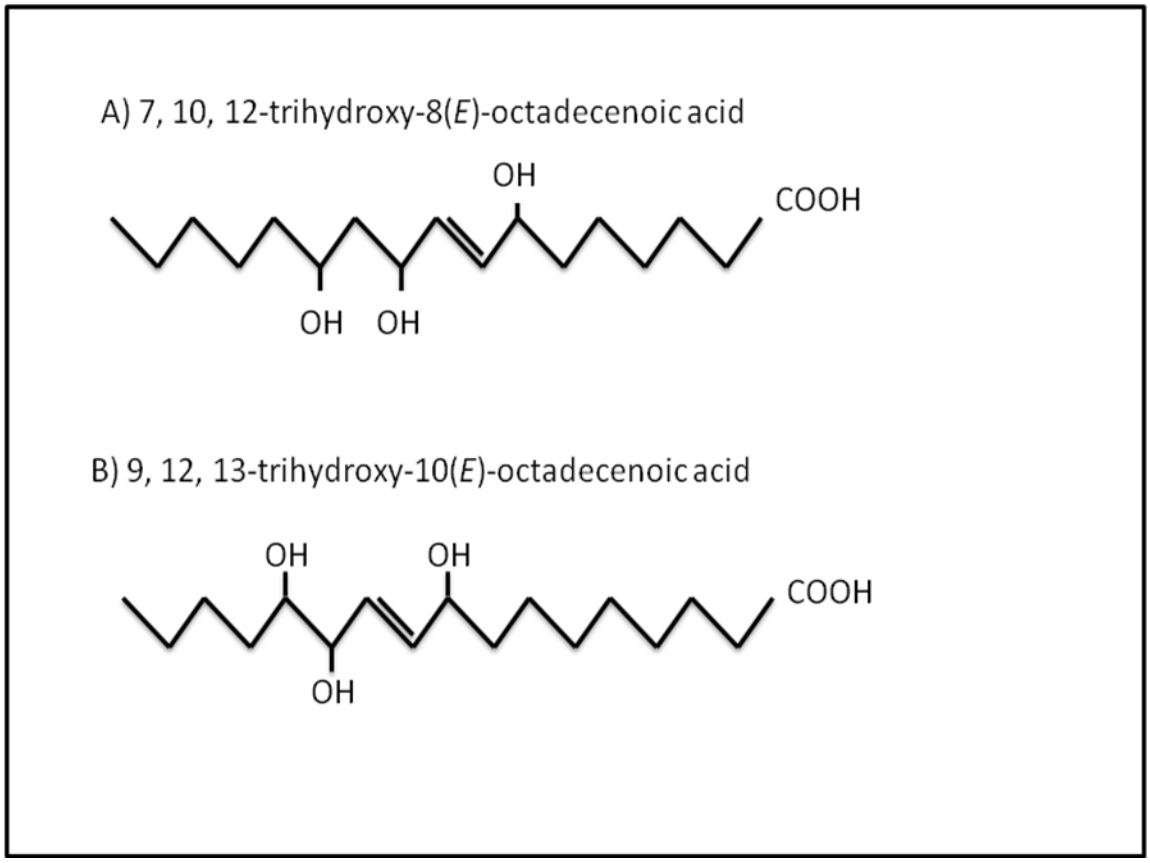


Figure 2.1 Comparison of TOD (A) and plant-derived fatty acids (B). The location of hydroxyl groups and double bonds appear to affect specificity of anti-fungal activity of trihydroxy fatty acids.

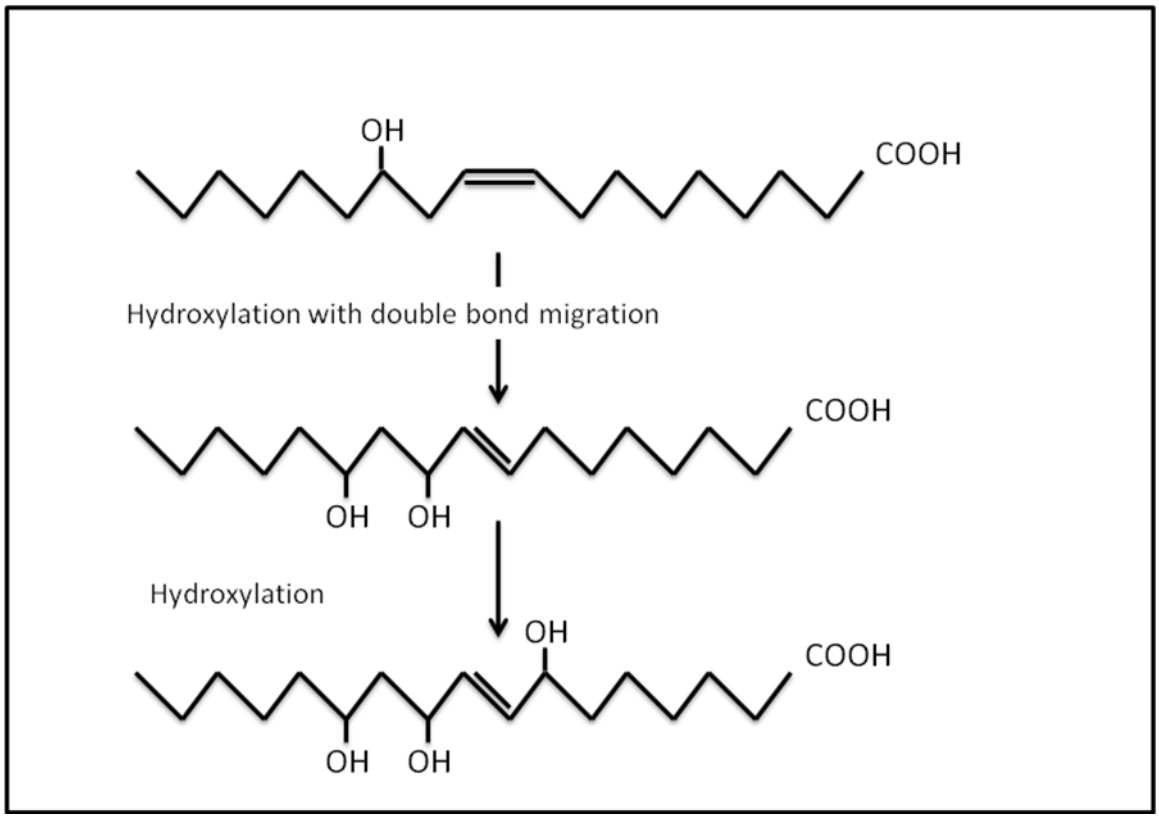


Figure 2.2 Proposed pathway for TOD conversion. The hypothetical conversion from ricinoleic acid to TOD illustrates the migration of the double bond and addition of two hydroxyl groups.

2.2. Materials and Methods

2.2.1. Chemicals

Ricinoleic acid was purchased from Nu-Chek-Prep, Inc. (Elysian, MN). DOD and TOD were obtained from Dr. Tsung-Min Kuo (Microbial Genomics and Bioprocessing Group, USDA, ARS, NACUR, Peoria, IL) and used as controls in fungal assays and as GC standards. Antibiotics were purchased from Sigma (St. Louis, MO).

2.2.2. Microorganisms

P. aeruginosa NRRL-23260 (TOD producer) and NRRL B-800 (non-TOD producer) were used as parents and control strains. Both NRRL-23260 and NRRL B-800 were gifts from Dr. Tsung-Min Kuo (Microbial Genomics and Bioprocessing Group, USDA, ARS, NACUR, Peoria, IL). When necessary, a laboratory strain of *P. aeruginosa*, PAO1, was used as a reference strain. All bacterial strains were routinely maintained on Luria-Bertani (LB) agar and in L-broth. For production of TOD, *P. aeruginosa* was grown in Wallen Fermentation broth, medium containing dextrose, yeast extract, K_2HPO_4, $MgSO_4$, $FeSO_4$, and H_2O (Kuo, *et al*., 2001). *Magnaporthe grisea* is a plant pathogenic fungus, and the most common cause of rice blast. *M. grisea* cultures were gifts from Dr. Jin-Rong Xu of Purdue University and maintained on potato dextrose agar (PDA) or in potato dextrose broth (PDB).

2.2.3. Construction of a random transposon insertion library

P. aeruginosa strain NRRL B-23260, an environmental isolate from an Illinois pig farm, which has a high rate of TOD conversion from ricinoleic acid was mutagenized with a transposon (Kuo and Nakamura, 2004). The transposon, carried on a plasmid pSUP102, was introduced from an *E. coli* host (S17-1 λ *pir*) (Simon, *et al*., 1989) to *P. aeruginosa* NRRL B-23260 strain via bi-parental conjugation (Figure 2.3). *E. coli* (S17-1 λ *pir*/Tn*5*-B21::pSUP102) was grown in Luria-Bertani (LB) broth supplemented with 20 µg/mL of gentamicin to maintain the plasmid. *P. aeruginosa* NRRL B-23260 (recipient) was grown in LB. Both the donor and the recipient were grown at 37°C with aeration. Conjugation was performed as previously described (Suh, *et al*., 1999). Briefly, 250 µL of *E. coli* donor grown to mid-log was mixed with 40 µL of *P. aeruginosa* recipient grown microaerobically at 42^{o}C with nitrate as the terminal electron acceptor. The cell mixture was spotted on a LB plate and incubated overnight at 30^{o}C to promote conjugation. The transconjugants that received random transposon insertions were selected on *Pseudomonas* Isolation Agar (PIA) supplemented with 100 µg/mL of tetracycline. Each Tc^{r} *P. aeruginosa* that represents a random transposon insertion mutant was transferred to 100 µL of LB supplemented with tetracycline in 96 well microtiter plates and grown overnight at 37^{o}C with shaking at 200 rpm. The following day, 100 µL of 10% skim milk was added to each well and each microtiter plate was stored at -80^{o}C until each mutant could be tested for TOD production.

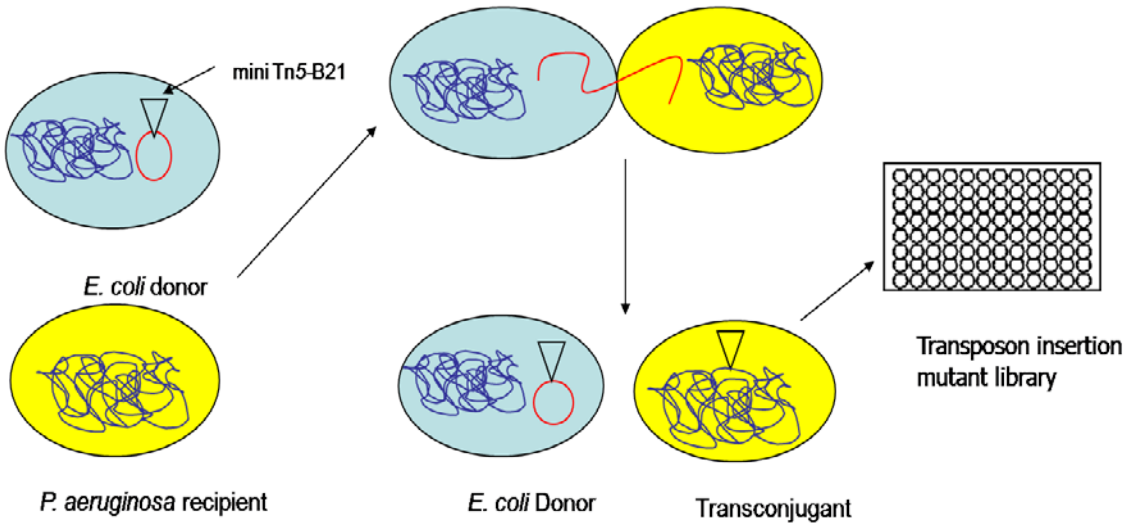


Figure 2.3 Construction of a random transposon insertion library. The *E. coli* donor (S17-1 λ *pir*) carried the plasmid pSUP102 with the transposon Tn*5*-B21 (Simon, *et al.*, 1989). TOD producing *P. aeruginosa* strain NRRL B-23260 was used as the recipient for generation of Tn*5* insertion library.

2.2.4. Development of a high throughput TOD bioassay (This assay was developed in collaboration with Dr. Katheryn Lawrence and Taylor Boozer Hatchett of the Plant Pathology Department, Auburn University).

M. grisea from freezer stock was plated onto PDA and grown at 30^{o}C for five to seven days. After initial growth, a "plug" from PDA with fungal growth was inoculated into 100 mL of potato dextrose broth (pH 7.0) and allowed to grow at 25^{o}C with shaking at 160 rpm for five days. To prepare the fungus for the bioassay, the five day old *M. grisea* culture grown in potato dextrose broth was blended for 30 seconds in fresh potato dextrose broth to separate the hyphae. Blended culture (200 µL) was then added to each well of a 96-well microtiter plate. Pure TOD, dissolved in 100% ethanol, in concentrations ranging from 0 µg/mL to 450 µg/mL was added to the wells in triplicate in 90 µg/mL increments to determine the minimum inhibitory concentration (MIC). Further testing was performed with concentrations from 90 µg/mL to 180 µg/mL by adding the stock TOD solution in 15 µg/mL increments. A final test for concentration was performed with concentrations ranging from 135 µg/mL to 150 µg/mL by adding the stock TOD solution in 3 µg/mL increments. Controls of 100% ethanol and distilled water were also added to the assay to determine the effect of diluents on the cultures. Fungal cultures were incubated at 30^{o}C for five to seven days and results were observed visually.

The minimum inhibitory concentration (MIC), 144 µg/mL, was used in a bioassay to test supernatants derived from *P. aeruginosa* NRRL B-23260 (TOD producing strain), *P. aeruginosa* NRRL B-800 (TOD non-producing strain), and *P. aeruginosa* PA01. To

prepare bacterial supernatants, overnight cultures grown in LB were used to inoculate 150 µl of Wallen-Fermentation broth (pH 7.0), growth medium in 96-well microtiter plates. This medium enhances the conversion of ricinoleic acid to TOD. The cells were incubated at 30°C with shaking at 180 rpm for 24 hours until they reached stationary phase (Kuo, *et al*., 2001). Under these conditions, *P. aeruginosa* should yield greater than forty-five percent conversion of RA to TOD (Kuo, *et al*., 2001). RA was added to final concentration of 0.5% with 7.5 µL of a 10% stock solution, or 5 µg/µL. Bacteria were grown for an additional 24 hours at 30°C with shaking during which microbial conversion was catalyzed (Kuo, *et al*., 2001). Following microbial conversion, cultures were centrifuged for 30 minutes at 7000 rpm, and supernatants collected and stored at -80^{o}C for use in a bioassay to test anti-fungal activity. Bacterial supernatants were initially tested in a bioassay using supernatants volumes ranging from 0 µL/mL – 200 µL/mL per well in increments of 25 µL/mL to determine the optimal volume to add to the fungal assay. Results were observed visually. It was determined that 7.5 µL of the parental supernatant consistently inhibited *M. grisea* growth. Thus, the volume of 7.5 µL was used to initially screen 15,168 Tn*5*-B21 insertion mutants to identify potential TOD^{-} mutants. This bioassay was developed in collaboration with Dr. Katheryn Lawrence and Taylor B. Hatchett. Specifically, I assisted in development of the bioassay by providing pure TOD and *P. aeruginosa* culture supernatants. In addition, I assisted in the set up of the assay and analysis of the bioassay data.

2.2.5. Identification of TOD-deficient *P. aeruginosa* Tn*5*-B21 insertion mutants

Each of the 15,168 Tn*5* insertion mutants was grown under conditions that are optimal for RA to TOD conversion (Kuo, *et al*., 2001). Transposon insertion mutants were taken from freezer stock 96-well microtiter plates and inoculated directly into 150 µL of Wallen-Fermentation broth (pH 7.0) in wells of 96-well microtiter plates. Following microbial conversion, all cultures in microtiter plates were centrifuged for 30 minutes at 7000 rpm and the supernatants were collected and stored at -80^{o}C for testing of anti-fungal activity. (Figure 2.4)

M. grisea was initially grown using conditions mentioned above and then sub-cultured into 100 mL of PDB. Fungal cultures were grown at 25^{o}C for 4 – 5 days using methods and conditions previously described; 200 µL of fungal culture was added to each well of a 96-well microtiter plate. To assess TOD production by each of the Tn*5* insertion mutants, 7.5 µL of each *P. aeruginosa* mutant supernatant (~169 µg/mL final concentration of TOD in each fungal assay well at 45% conversion rate) was added and mixed to *M. grisea* fungal cultures in wells of microtiter plates. The supernatant and fungal mixture was then incubated at 25^{o}C for 4 – 5 days. Presence or absence of TOD activity in *P. aeruginosa* culture supernatants was determined visually by assessing presence or absence of fungal growth (Figure 2.5). Each Tn*5* insertion mutant was tested in triplicate and compared to the culture supernatants of the parental strain *P. aeruginosa* NRRL B-23260.

The presence or absence of TOD in the culture supernatants of putative mutants was assayed by gas chromatography (GC). The culture supernatants were collected and extracted with ethyl acetate/methanol (9:1) and acidified to pH 2 with 6N HCl. Solutions were mixed by inversion and centrifuged for 1 minute at 13,200 rpm. The ethyl acetate/methanol extraction was repeated a total of three times and was precipitated by evaporation of the organic solvent as previously described (Bae, *et al*., 2007; Chang, *et al*., 2007). Dried pellets containing fatty acids were resuspended in 500 µL of hexanes/methyl-tert-butyl ether (1:1) and were analyzed via GC on a 25 m x 0.2 mm phenyl methyl siloxane fused silica capillary column. The GC analysis was performed by John McInroy of the Department of Plant Pathology at Auburn University.

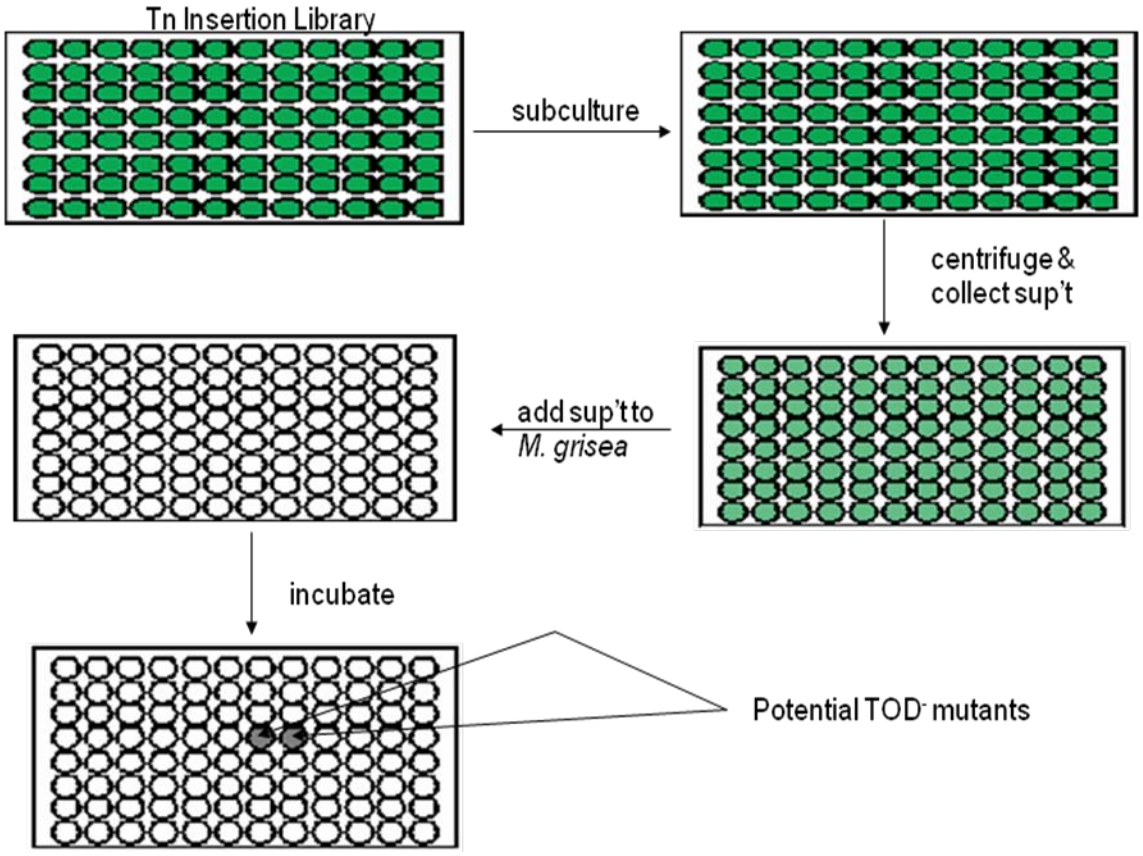


Figure 2.4 Identification of TOD$^-$ mutants. *P. aeruginosa* supernatants of transposon insertion mutants were assayed for antifungal activity against *M. grisea*. Those that failed to inhibit the fungal growth were designated as TOD$^-$ mutants.

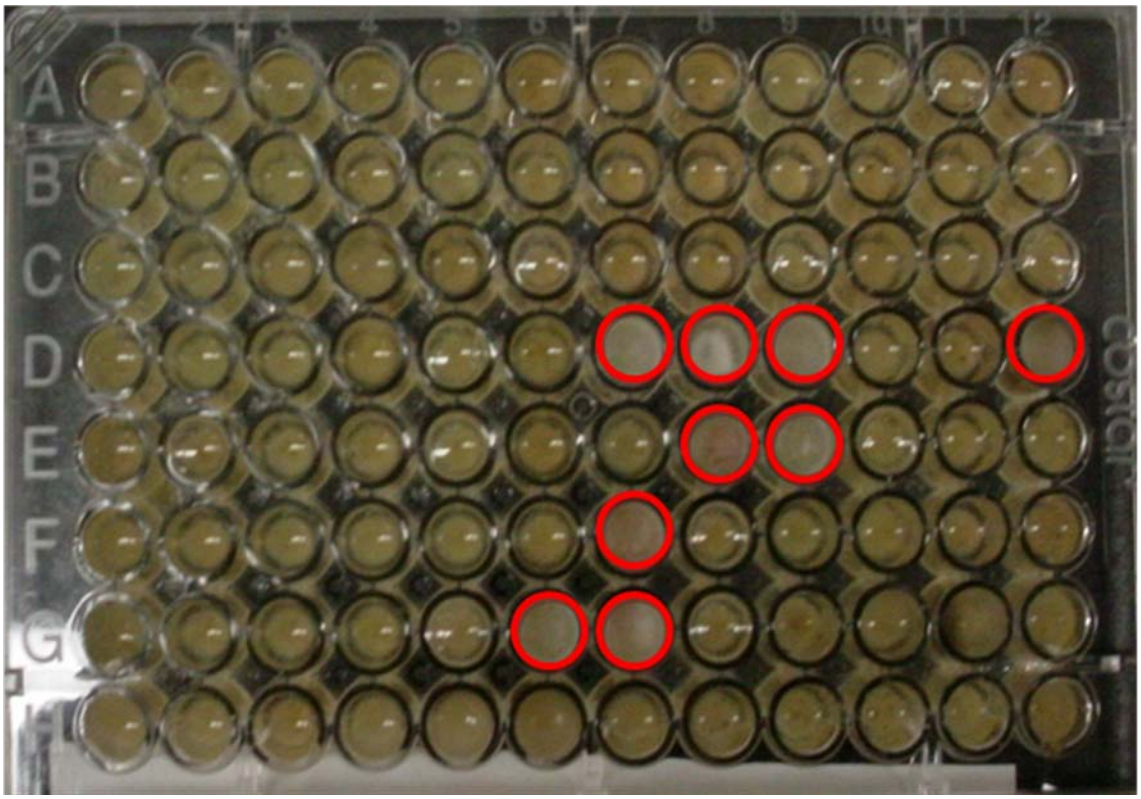


Figure 2.5 Visual observations of fungal growth

TOD^- mutants (circles) are wells with white/pink film of growth at surface of well; TOD^+ mutants are wells with hyphae at the bottom of the well and no apparent growth of fungus on the surface.

2.3. Results and Discussion

2.3.1. Construction of a random transposon insertion library of *P. aeruginosa* NRRL B-23260

In order to identify *P. aeruginosa* genes involved in TOD production, I performed a transposon mutagenesis to isolate mutants that can no longer convert ricinoleic acid to TOD. A derivative of Tn*5* (Tn*5*-B21) (Simon, *et al*., 1989) was used which yielded 15,168 tetracycline resistant (Tc^r) random insertion mutants of *P. aeruginosa* strain NRRL B-23260. Each transposon insertion mutant was grown in LB supplemented with 100 µg/mL of tetracycline and stored at -80°C until tested for TOD production in the antifungal bioassay. *P. aeruginosa* contains approximately 5800 genes and thus ~15,000 mutants should give an almost 3-fold coverage of the genome. With overlapping coverage it was surmised that mutation would occur in both the biosynthetic as well as regulatory genes involved in TOD production.

2.3.2. Development of a high throughput TOD bioassay

In order to facilitate genetic screening of ~15,000 transposon insertion mutants of *P. aeruginosa*, a high throughput assay to assess the antifungal activity present in the culture supernatants of bacteria was developed. This assay utilizes 96-well microtiter plates and can be performed using only a growth incubator. As an initial step, the assay was developed using purified TOD to assess the biological activity range of the fatty acid. In the initial MIC (minimum inhibitory concentration) test, complete inhibition of growth for *M. grisea* was observed with 180 µg/mL final concentration of

TOD. By varying the range and using smaller increments as described in Materials and Methods, an MIC of TOD was determined. The data from the MIC tests demonstrated that for the 96-well microtiter plate assay, a concentration of 144 µg/mL of TOD completely inhibited the growth of *M. grisea* (Table 2.1).

The high throughput bioassay was developed to facilitate genetic screening of *P. aeruginosa* mutants defective for TOD production. The bioassay was optimized with *P. aeruginosa* culture supernatants. *P. aeruginosa* was grown in 150 µL of Wallen Fermentation broth in 96-well microtiter plates under TOD conversion promoting conditions as described by Kuo *et al*. (2001). With addition of 0.5% ricinoleic acid, it was determined that 7.5 µL of the parent strain (NRRL B-23260) consistently inhibited the growth of *M. grisea* in the bioassay. At 45% conversion rate of ricinoleic acid to TOD (Kuo *et al*., 2001), this equates to addition of approximately 169 µg/mL of TOD in each assay. (Table 2.2) Conversely, since it was determined that MIC with purified TOD was 144 µg/mL, it is possible that the conversion rate in our growth conditions in microtiter plate wells is less than the optimal at ~38%.

2.3.3. Identification of TOD-deficient *Pseudomonas aeruginosa* Tn*5*-B21 mutants

Due to the fact that *P. aeruginosa* was previously shown to produce TOD after growth in microtiter plates, the genetic screen and results from GC provided the means to identify TOD biosynthetic genes. Because *P. aeruginosa* NRRL B-23260, as previously discussed, produces the highest yield of TOD, I anticipated that the anti-fungal activity

Table 2.1 Inhibitory concentration of TOD (µg /mL) against *Magnaporthe grisea* using the microtiter plate assay*

	TOD Concentration (µg /mL)					
	142.8	145.0	148.3	150.0	153.6	156.0
M. grisea	1	1	1	0	0	0

0 = no mycelial growth
1 = mycelial growth

*Done in collaboration with Dr. Katheryn Lawrence and Taylor Boozer Hatchett of the Plant Pathology Department at Auburn University.

Table 2.2 *Magnaporthe grisea* mycelial growth as affected by supernatants from *Pseudomonas aeruginosa* strains NRRL B-800 (800) and NRRL B-23260 (23260) at increasing volumes*

	Volume of Supernatant Added (µL/mL)										
Strain	0	24	47	69	90	111	130	148	166	183	200
800	1	1	1	1	1	1	1	0	0	0	0
23260	1	1	0	0	0	0	0	0	0	0	0

0 = no mycelial growth
1 = mycelial growth

*Done in collaboration with Dr. Katheryn Lawrence and Taylor Boozer Hatchett of the Plant Pathology Department at Auburn University.

and relative extent of *M. grisea* inhibition displayed in the assay was due to the amount of TOD obtained from culture supernatants. Therefore, any mutant that failed to inhibit the growth of *M. grisea* would suggest that the transposon insertion mutant was deficient in TOD production. Some transposon insertion mutants may still produce TOD but in quantities too miniscule to affect the growth of *M. grisea* in the bioassay.

Each of the 15,168 Tn*5* insertion mutants of *P. aeruginosa* strains NRRL B-23260 were tested for TOD production in the 96-well microtiter plate assay to identify mutants defective for conversion of RA to TOD. *P. aeruginosa* was grown in Wallen Fermentation broth under conditions that promote TOD production and 7.5 µL of supernatant from each mutant was added to 200 µL of the fungal culture to assess inhibition of *M. grisea* growth (Figure 2.4). The assay was performed in triplicate. For the first screen, any *P. aeruginosa* was scored as a potential mutant that showed even a slight decrease in *M. grisea* growth in any one of three wells of the triplicate assay. Due to low stringency, the initial genetic screen resulted in identification of approximately 2000 putative mutants. In subsequent screens, the stringency was increased and only those *P. aeruginosa* mutants that produced supernatants which severely decreased the fungal growth in all three wells were scored. From these genetics screens, 28 transposon insertion mutants were identified and designated as TOD^-. The validity of these mutants was verified in subsequent bioassays. Our bioassay does not distinguish between identification of *P. aeruginosa* mutants that are affected in biosynthetic genes versus

regulatory genes. Thus, biosynthetic and regulatory genes involved in TOD production in *P. aeruginosa* may be represented in the pool of 28 mutants.

Based on the proposed pathway, it was expected that a lipoxygenase and/or monooxygenase was probably involved in TOD conversion. The *P. aeruginosa* genome encoded for one probable lipoxygenase (*PA1169*) whose function has not yet been demonstrated. The bacterium also contains at least 11 and as many as 54 genes that may be involved in encoding for a monooxygenase. We expect some of our mutants have a transposon inserted into some of these genes. This will allow the assignment of physiological functions to some of these genes including those that encode for hypothetical proteins.

2.3.4. Verification of *P. aeruginosa* TOD$^-$ mutants via gas chromatography

Because *P. aeruginosa* produces multiple virulence factors that could potentially affect *M. grisea* growth, any transposon insertion mutant that failed to inhibit the fungal growth may have a defect in genes other than for TOD production. The amount of TOD produced by the twenty eight *P. aeruginosa* TOD$^-$ mutants isolated was determined via gas chromatography. Fatty acids in the supernatants of each TOD$^-$ mutant were extracted as previously described using ethyl acetate and resolved via GC. As controls, *P. aeruginosa* NRRL B-23260 (parent strain), NRRL B-800 (non-TOD producer), and PA01 (a laboratory strain) were also analyzed to determine relative TOD levels produced by the insertion mutants. As illustrated in Table 2.3, all twenty eight TOD$^-$ mutants,

showed severe decrease in the peak corresponding to TOD. The GC data confirmed that 28 TOD$^-$ mutants identified by our bioassay were reduced for TOD production. By combining the results from the bioassay and GC analysis, I successfully identified 28 transposon insertion mutants deficient in TOD production were identified that could be utilized for transposon insertion gene identification.

Table 2.3 GC analysis of 28 TOD$^-$ mutants

	Retention time	Response*
P1A1	17.885	146341
P1B12	17.884	106256
P1C2	17.883	135109
P1C8	17.882	103956
P1D2	17.877	56567
P1D5	17.880	136803
P1D9	17.891	146038
P1D12	17.890	142836
P1E2	17.890	89338
P1F2	17.887	50636
P1F3	17.892	187470
P1F5	17.892	122298
P1F10	17.892	131442
P2A12	17.888	136631
P2B12	17.893	138358
P2C12	17.893	149774
P2E2	17.891	11352
P2F11	17.897	138542
P2F12	17.896	193678
P2H11	17.896	159745
P3A1	17.898	150827
P3A2	17.890	77513
P3A4	17.890	94152
P3B2	17.887	102138
P3B4	17.886	74933
P3B9	17.891	73581
P3C7	17.896	104753
P3C12	17.899	76100
NRRL B-23260	17.914	1149524
NRRL B-800	17.910	4535

*Response: relative amount of TOD produced

2.3.5. Summary

A high throughput bioassay that tests for antifungal activity of a novel antifungal agent, 7, 10, 12-trihydroxy-8(*E*)-octadecenoic acid (TOD), was developed to facilitate isolation of *P. aeruginosa* mutants that are defective for TOD production. This assay utilizes 96 well microtiter plates and requires only pipettors and an incubator. We determined that the MIC of pure TOD on *M. grisea* was 144 µg/mL. We then screened 15,168 transposon insertion mutants of *P. aeruginosa* strain NRRL B-23260 and 28 mutants were identified that appeared to be defective for inhibiting the growth of *M. grisea*, thus suggesting that they were defective for TOD production. The inability of these 28 mutants to produce TOD in sufficient quantity to inhibit *M. grisea* growth was confirmed by gas chromatography analysis in which the TOD present in bacterial supernatants were determined. Isolation of TOD^- mutants presents the initial step towards elucidating the molecular mechanisms for microbial conversion of RA to TOD by *P. aeruginosa.* Identification of genes involved in TOD production is a critical step in constructing an economically competitive and environmentally friendly biological crop protection agent.

CHAPTER 3

IDENTIFICATION OF *Pseudomonas aeruginosa* GENES INVOLVED IN TOD PRODUCTION

3.1. Introduction

Many secondary metabolites produced by microbial organisms have biological activities that can be utilized as antimicrobial agents, anti-cancer agents, or other pharmaceutical compounds (Bibb, 2005). Some microbial metabolites have been of great interest as potential plant defense agents because they can overcome the issue of pollution caused by overuse of synthetic chemical pesticides (Prabavathy, *et al*., 2006). These microbial metabolites are biodegradable and therefore do not accumulate in the environment to cause pollution (Prabavathy, *et al*., 2006). One such metabolite, a novel trihydroxy fatty acid, 7, 10, 12-trihydroxy-8(*E*)-octadecenoic acid (TOD), has shown to have the potential of being an effective biological control agent (Kuo and Nakamura, 2004).

A survey of TOD was conducted to test its biological activity against fungi, bacteria, and nematodes. The assessment of biological activity of TOD against various fungi demonstrated that TOD's antifungal activity was highly specific for *M. grisea*, the

causative agent of rice blast disease (Hatchett, *et al*., 2009; Kuo and Nakamura, 2004). The investigators also demonstrated that TOD was active against many soil bacteria with no species specificity (Hatchett, *et al*., 2009). At low concentrations, TOD did not inhibit the growth of other pathogenic fungi or nematodes.

TOD is produced by the gram-negative bacterium, *Pseudomonas aeruginosa*, from the monohydroxy fatty acid, ricinoleic acid found in castor bean oil (Kuo and Nakamura, 2004). The hypothesized conversion of ricinoleic acid to TOD involves two steps: 1) the hydroxylation of two separate carbon atoms, C-7 and C-10, which results in the rearrangement of the double bond from C 9–10 (*cis*) to C 8–9 (*trans*); and 2) hydroxylation of the secondary dihydroxy fatty acid at C-12 results in TOD which has proven to be an effective antifungal agent (Figure 3.1) (Kato, *et al*., 1986; Kuo and Nakamura, 2004).

Based on the specificity of TOD against *M. grisea*, it is an ideal compound to be used agriculturally to combat this important agricultural pest to reduce world hunger. The ultimate goal of this research is to facilitate the production and isolation of TOD to make it economically feasible to utilize as an antifungal agent. To achieve this aim, it is desirable to convert *P. aeruginosa* via genetic and metabolic engineering to a microbial factory. However, to achieve this goal, it is first imperative to identify the genes and the pathways involved in TOD production, including the biosynthetic and regulatory pathways. As an initial step, a genetic approach was taken to identify the genes involved

in TOD production in *P. aeruginosa*. As described in the previous chapter, we isolated 28 transposon insertion mutants that are defective for TOD production. Out of 28 TOD^- mutants, I focused on mapping 11 mutants because they appeared to have single transposon insertions.

Based on the predicted pathway of fatty acid hydroxylation (Figure 3.1), I expected that genes that encode for enzymes such as monooxygenase or lipoxygenase would be found. These are enzymes involved in hydroxylation of fatty acids or lipids. In addition, I expected that genes involved in either uptake or excretion of TOD would be identified, especially if the molecule requires a specific transport system. Furthermore, I hoped to identify any regulatory functions for TOD production. Lipoxygenase is characterized as non-heme iron containing dioxygenase that oxygenates polyunsaturated fatty acids (Vidal-Mas, *et al*., 2005). A monooxygenase is an enzyme with the ability to add molecular oxygen from an oxygen atom to a substrate following subsequent reduction of a second oxygen atom to water (Prigge, *et al*., 1997). Also of importance would be genes involved in the transport of TOD, which in *P. aeruginosa* could account for approximately 400 genes (Stover, *et al*., 2000).

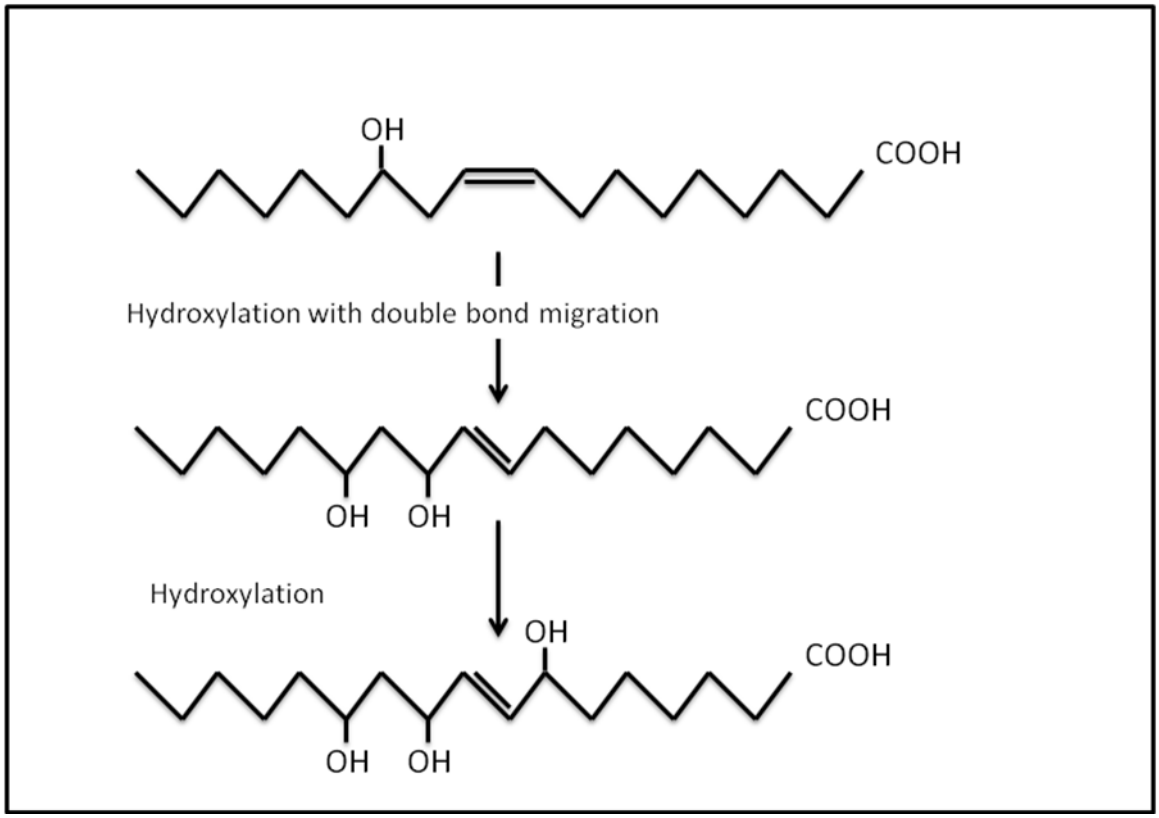


Figure 3.1 Proposed pathway for TOD conversion. The hypothetical conversion from ricinoleic acid to TOD illustrates the migration of the double bond and addition of two hydroxyl groups.

3.2. Materials and Methods

3.2.1. Chemicals

Ricinoleic acid was purchased from Nu-Chek-Prep, Inc. (Elysian, MN). DOD and TOD were obtained from Dr. Tsung-Min Kuo (Microbial Genomics and Bioprocessing Group, USDA, ARS, NACUR, Peoria, IL) and used as controls in fungal assay and as GC standards. Antibiotics were purchased from Sigma (St. Louis, MO) and used at the following concentrations: Ampicillin, 100 µg/mL for *Escherichia coli*; carbenicillin, 150 µg/mL and/or 250 µg/mL for *P. aeruginosa*; gentamicin, 20 µg/mL for *E. coli* and 100 µg/mL for *P. aeruginosa*; tetracycline, 20 µg/mL for *E. coli* and 100 µg/mL for *P. aeruginosa*; nalidixic acid, 20 µg/mL for *E. coli*. 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) was purchased from Biosynth AG (Switzerland) and used at 40 µg/mL. Restriction enzymes, *Taq* polymerase, and T4 DNA ligase were purchased from New England Biolabs (Ipswich, MA). Proofing reading thermostable polymerase, *Pfu*, was purchased from Stratagene (La Jolla, CA). Oligonucleotide primers were purchased from Integrated DNA technologies (Coralville, IA).

3.2.2. Microorganisms

P. aeruginosa NRRL-23260 (TOD producer) and NRRL B-800 (non-TOD producer) were used as parents and control strains. Both NRRL-23260 and NRRL B-800 were gifts from Dr. Tsung-Min Kuo (Microbial Genomics and Bioprocessing Group, USDA, ARS, NACUR, Peoria, IL). When necessary, a laboratory strain of *P. aeruginosa*, PAO1, was used as a reference strain. All bacterial strains were routinely

maintained on Luria-Bertani (LB) agar and in L-broth. For production of TOD, *P. aeruginosa* were grown in Wallen Fermentation broth, medium containing dextrose, yeast extract, K_2HPO_4, $MgSO_4$, $FeSO_4$, and H_2O (Kuo, *et al*., 2001).

3.2.3. Genetic mapping of Tn*5*-B21 insertions

Several different approaches were used to identify the transposon insertion sites, including cloning Tn*5* and flanking sites, arbitrary PCR of Tn*5*, Rapid Amplification of Transposon Ends PCR (RATE PCR) and Thermal Asymmetric Interlace PCR (TAIL PCR).

3.2.3.1. Southern blot analysis

Total genomic DNA was isolated from *P. aeruginosa* using the Wizard Genomic DNA Isolation System (Promega, Madison, WI) according to the manufacturer's recommendation. Genomic DNA was digested with either *Eco*RI or in combination of *Eco*RI with either *Pst*I, *Kpn*I, *Bam*HI, *Sal*I, or *Sac*I, separated on 1% agarose gel, transferred to nitrocellulose paper by capillary action, and probed with PCR amplified *tetAB* genes of Tn*5*-B21. The 1.2 Kb *tetAB* fragment was PCR amplified from *E. coli* carrying pSUP102, which contains the mini-Tn*5*-B21 (Simon, *et al*., 1989), with *Pfu* DNA polymerase. (Figure 3.2) Five hundred nanograms of the DNA probe were labeled with biotin using the Ambion's BrightStar® Nonisotopic Detection System according to the manufacturer's instructions (Ambion, Austin, TX). The hybridization was performed

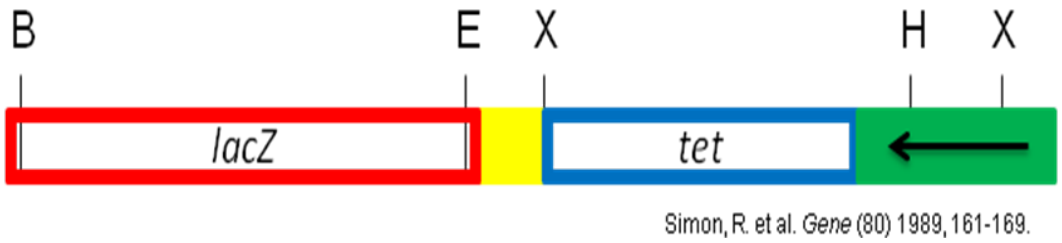


Figure 3.2 Tn*5*-B21 restriction endonuclease map (not drawn to scale)

B:*Bam*HI, E:*Eco*RI, X:*Xho*I, H:*Hind*III

at 42^{o}C overnight with shaking and the membrane was washed with 2X SSC/0.1% SDS then with 0.1X SSC/ 0.1% SDS. Hybridized probe was then detected with the Ambion's BrightStar BioDetect kit, using instructions provided by the kit, which includes a streptavidin-alkaline phosphatase conjugate and a light emitting substrate (CDP-Star^{TM}). Binding of the labeled probe to the DNA fragment was visualized on Kodak x-ray film and analyzed for presence of *tetAB* genes, single or multiple insertion sites, and relative size of the gene with the Tn*5* insertion.

3.2.3.2. Cloning of Tn*5* and flanking sequences

To clone the transposon and the flanking sequences, 5 µg of genomic DNA was digested with appropriate restriction endonucleases and ligated into the plasmid vectors, pUC19 or pSS213 (Suh, *et al*., 2004) that were digested with the same restriction endonucleases as the genomic DNA inserts. Ligations were performed at 16°C with T4 DNA ligase (NEB, Ipswich, MA). The ligation mixtures were electroporated into *E. coli* DH10B (Stratagene, La Jolla, CA) using a standard procedure (Ausubel, *et al*., 1987). Transformants carrying *tetAB* genes of Tn*5*-B21 plus the flanking DNA sequences of the genomic DNA were selected on LB agar with tetracycline (20 µg/mL) and X-gal (40 µg/mL), as Tc^{r}, *lac*$^{-}$ white colonies. Tc^{r} transformants were analyzed via PCR amplification with appropriate primers and restriction mapping. Following verification, the Tn*5*-B21 clones were sequenced with a Tn*5* specific oligonucleotide primer to determine the sequence of the DNA flanking the transposon site.

3.2.3.3. PCR approaches

Arbitrary PCR was performed as previously described (O'Toole and Kolter, 1998 (A)). Primers used for the first round of arbitrary PCR were Tn*5*Ext, specific to Tn*5*, and ARB1 and/or ARB6, random primers (O'Toole and Kolter, 1998 (A)) (Table 3.1). Nested primers Tn*5*Int, identical to the 3' end of Tn*5*Ext, and ARB2, identical to the 5' end of ARB1 and ARB6, were used in the second round to amplify the fragments for DNA sequencing. PCR products were separated on agarose gels, purified and sequenced with the Tn*5*Int primer. (Figure 3.3)

RATE PCR was performed as previously described (Karlyshev, *et al*., 2000) using the Tn*5*Ext (same primer used for arbitrary PCR) as the primer. The first round of PCR utilized a 50^{o}C annealing temperature for binding of the primer to the transposon and linear amplification of DNA. The second round employed a 30^{o}C annealing temperature for non-specific binding of the primer to flanking sequences. In the third round of RATE PCR, the annealing temperature was again raised to 50^{o}C to amplify products obtained from round two (Karlyshev, *et al*., 2000). PCR products were visualized on agarose gels and DNA bands were isolated for sequencing. Tn*5*Ext was used for sequencing of DNA fragments.

The three rounds of TAIL PCR were performed with Tn*5*Ext for the first, Tn*5*Int for the second and third rounds. In each PCR reaction, one of the nine arbitrary primers described in Qian, *et al*. (2005) were used in conjunction with the Tn*5* primers. (Table

Table 3.1 Tn*5* specific primers used in arbitrary PCR and cloning of sequences

Primer	Sequence (5’ to 3’)	Tm	% GC
Tn5Ext	GAA CGT TAC CAT GTT AGG AGG TC	51^{o}C	47.8
Tn5Int	CGG GAA AGG TTC CGT TCA GGA CGC	67^{o}C	62.5

This table demonstrates respective melting temperatures and GC percentages of each primer.

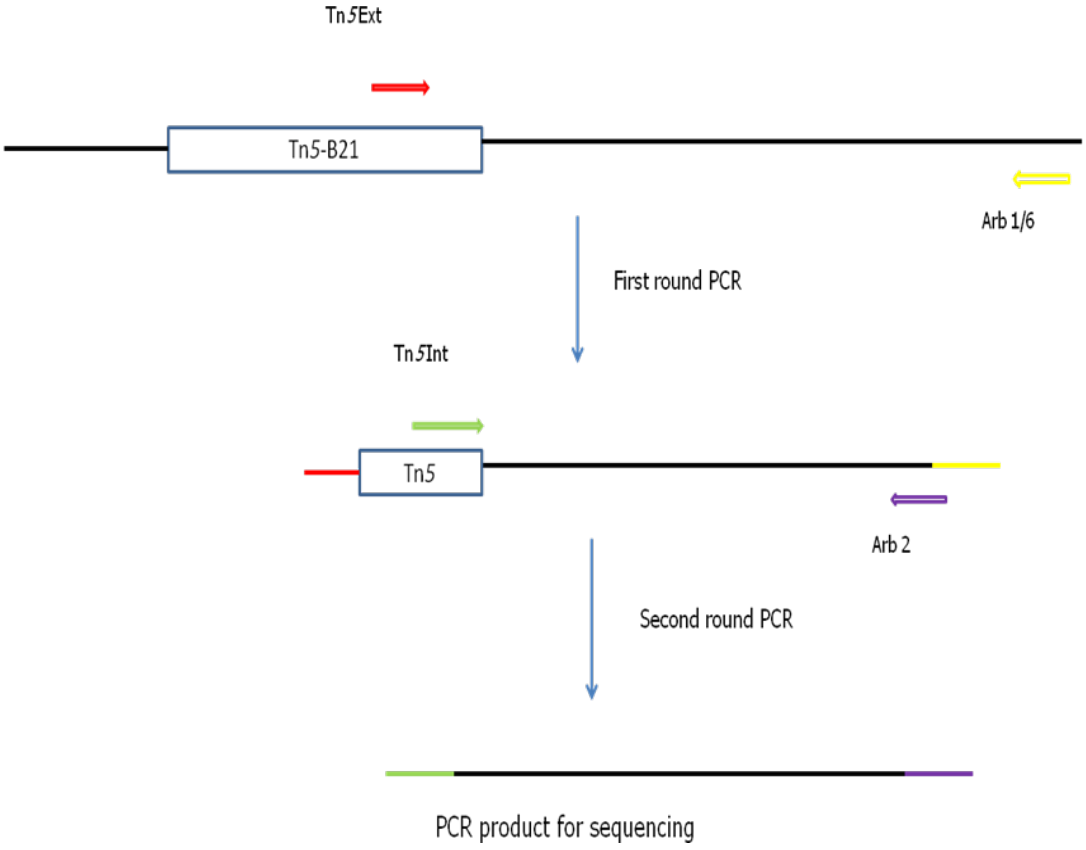


Figure 3.3 Schematic of arbitrary PCR

Table 3.2 TAIL PCR primers

TAIL PCR Arbitrary Primer Sequences*	
AD1	NTCGASTWTSGWGTT
AD2	NGTCGSWGANAWGAA
AD4	TGWGNANCASAGAGC
AD5	AGWGNAGWANCAWAGG
AD6	GAWCGICNGAIASGAA
AD7	TCSTICGNACITWGGA
AD8	STTGNTASTNCTNTGC
AD10	TCTTICGNACITNGGA
AD11	TTGIAGNACIANAGG

*Standard IUB/IUPAC nucleic acid codes

3.2) In the first round of TAIL PCR, the amplification was performed for a total of 22 cycles with annealing temperatures ranging from 25°C to 61°C for 15 seconds each and temperature ramping from 25°C to 72°C over 3 minutes to increase the specificity of the primer. Fifteen total cycles were used in the second round of PCR again with annealing temperatures ranging from 48°C to 61°C for 20 seconds each. The third round underwent the same conditions as the second round, which was used to further amplify PCR products (Qian, *et al*., 2005).

3.2.3.4. DNA Sequencing and other molecular and microbiological techniques

DNA sequencing was performed by the Auburn University Genomics and Sequencing Lab. DNA oligonucleotides were designed with the Lasergene DNAStar Primer Select software (Madison, WI). Plasmids were introduced into *P. aeruginosa* by tri-parental conjugation as previously described (Suh, *et al.*, 1999) using *E. coli* donor carrying the plasmid of interest, *E. coli* helper carrying the helper plasmid (pRK2013), and *P. aeruginosa* recipient. Transconjugants were selected on *Pseudomonas* Isolation Agar (PIA) supplemented with the appropriate antibiotic.

Growth curves were performed in 96-well microtiter plates in the BioTek Synergy HT (BioTek Instruments, Inc., Winooksi, VT) at 30°C or 37°C for 36 hours with shaking. Samples were read at OD_{600} every 17.5 minutes. Each well contained 150 µL of LB supplemented with appropriate antibiotics.

3.3. Results

3.3.1. Genetic mapping of Tn*5*-B21 insertions

In order to identify the genes involved in TOD production, I mapped the transposon insertion sites in each of the TOD$^-$ mutants that were identified in Chapter 2. I tried several different approaches to identify the transposon insertion sites, including cloning Tn*5* and flanking sites, arbitrary PCR of Tn*5*, RATE PCR and TAIL PCR.

3.3.1.1. Southern blot analysis

I did a Southern blot analysis (Southern, 1975) of the transposon mutants obtained from the bioassay to verify that these mutants contained the Tn*5*-B21 insertions and that they did not have multiple insertions of the transposon. Genomic DNA of the twenty eight TOD$^-$ mutants was digested with *Eco*RI, to maintain the *tetAB* gene for probing in the first round of Southern blotting. Twelve out of twenty-eight TOD$^-$ mutants showed a single, definitive, bright band when probed with *tetAB* sequence. Genomic DNA of these twelve TOD$^-$ mutants were then digested with *Eco*RI/ *Kpn*I, and Southern blots showed that of the eleven tested (P1A1 was digested with *Eco*RI/*Sal*I) in a second round of Southern blotting, only ten of the transposon insertion mutants gave single, definitive bright bands. The relative sizes of the DNA fragments carrying the transposon insertions were determined from this analysis (Table 3.3, Figures 3.4 and 3.5). It is not yet clear whether the other 17 TOD$^-$ mutants have multiple insertions or the genomic DNA was incompletely digested. Therefore, based on the data, I focused on mapping transposon insertions in the 11 TOD$^-$ mutants that showed a single fragment with *tetAB*.

Table 3.3 Results of Southern blot

	*Eco*RI/ *Kpn*I (size in kilobases)
P1A1	8.5 (*Eco*RI/ *Sal*I)
P1B12	8
P1D2	8
P1E2	8
P1F2	7
P2E2	7.5
P2H11	8
P3A1	7
P3A2	7
P3A4	7
P3C7	8

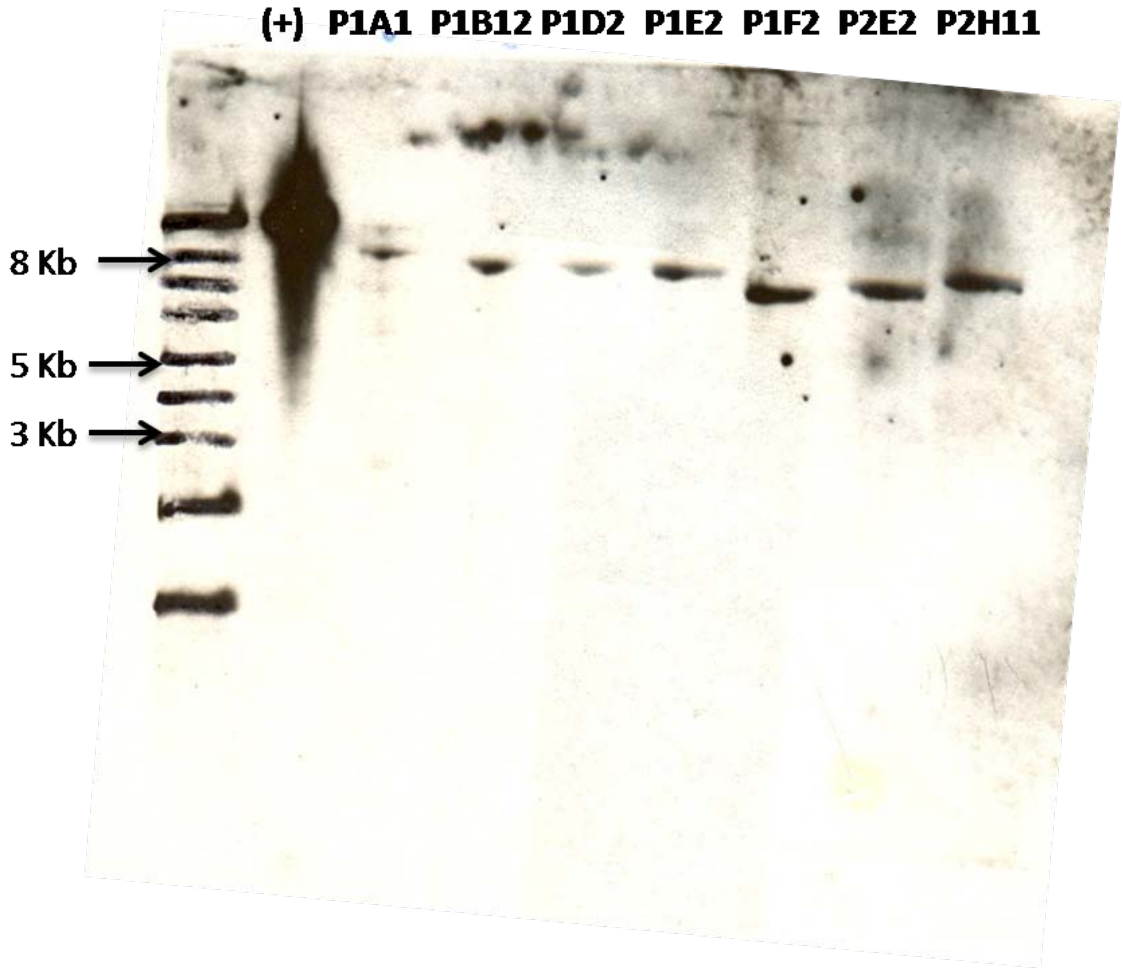


Figure 3.4 Southern analysis of 11 TOD$^-$ to determine relative size of digested DNA fragments carrying the transposon

(+): undigested genomic DNA of a Tn*5* insertion mutant

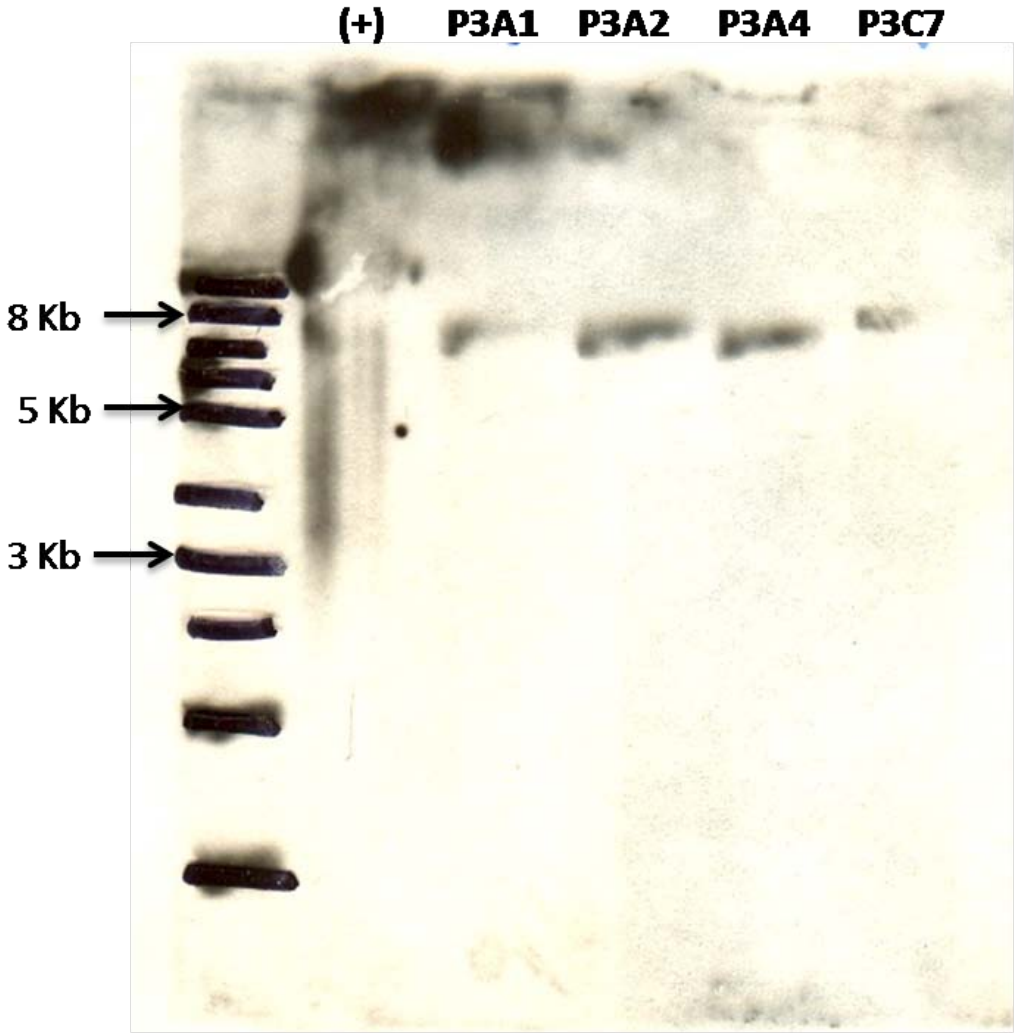


Figure 3.5 Southern analysis of 11 TOD$^-$ to determine relative size of digested DNA fragments carrying the transposon

(+): undigested genomic DNA of a Tn*5* insertion mutant

The eleven TOD$^-$ mutants with a single transposon insertion identified from Southern blot analyses were tested to determine if their TOD$^-$ phenotype was the result of growth defects. The cells were grown in 96-well microtiter plate at 37°C with shaking and their growth was monitored at OD_{600} for 36 hours. As demonstrated in Figures 3.6, 3.7, and 3.8, none of the eleven TOD$^-$ mutants demonstrated significant growth defects. The data suggest that the TOD$^-$ mutant phenotype in these eleven mutants are due to inactivation of genes involved in TOD production and not in general metabolic genes for promoting and maintaining growth under the tested conditions.

3.3.1.2. Cloning of Tn*5* and flanking sequences

In order to determine transposon insertion site, I attempted to clone the *tetAB* gene of the transposon along with the flanking sequence of the transposon insertion site for each mutant. The genomic DNA from eleven transposon mutants were digested with *Eco*RI and cloned into the *Eco*RI digested pUC19 plasmid. *Eco*RI cuts once immediately upstream of the *tetAB* genes and therefore separates Tn*5*-B21 into two DNA fragments. The putative clones were selected as Tc^r colonies and analyzed via PCR, restriction mapping, and DNA sequencing. The *tetAB* genes and the transposon flanking sequence were successfully cloned as an *Eco*RI fragment from five (P1A1, P1B12, P1D2, P2H11, and P3C7) out of eleven TOD$^-$ mutants. The mutant designation is from the microtiter plate number and position of the well in the microtiter plate. For one TOD$^-$ mutant (P1E2), the *tetAB* genes and the transposon flanking sequence was cloned as an *Eco*RI/ *Kpn*I fragment. The cloned insert DNA was sequenced using the Tn*5*Ext primer and the

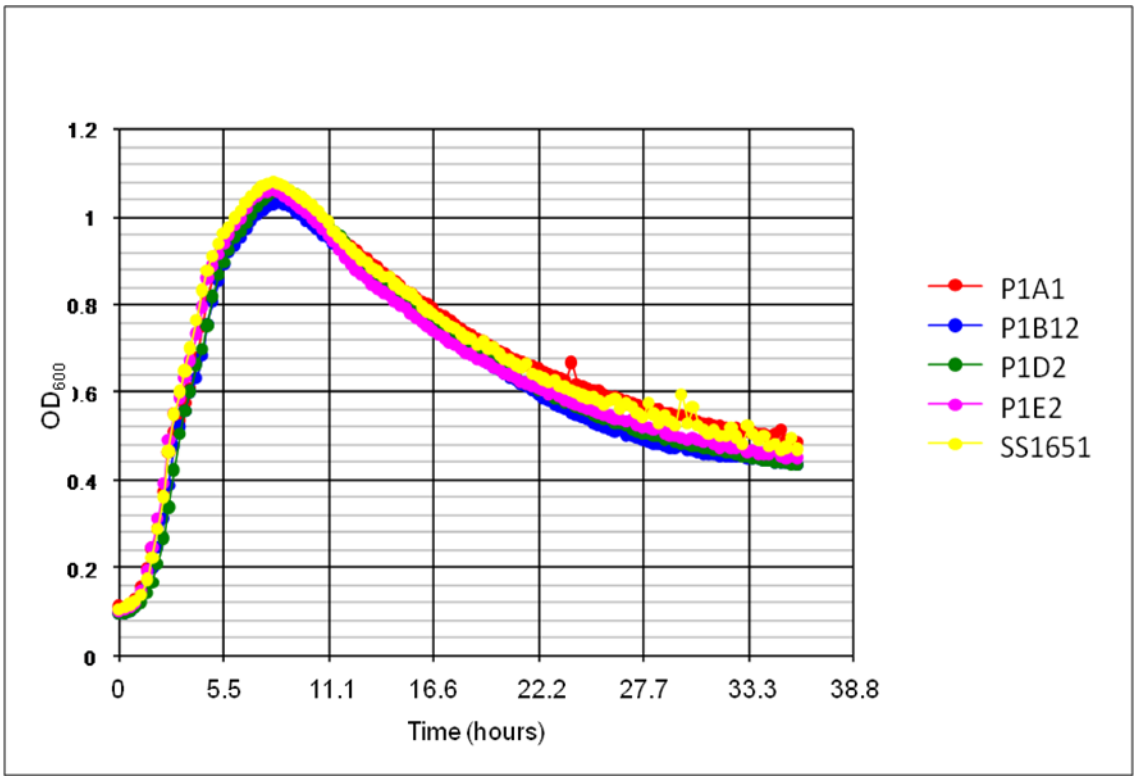


Figure 3.6 Growth curves of 11 TOD$^-$ (A). Cells were grown in 96 well microtiter plates at 37°C with shaking and OD_{600} was measured in a BioTek Synergy HT microplate reader. Growth curves were repeated 3 times.

*SS1651: *P. aeruginosa* NRRL B-23260

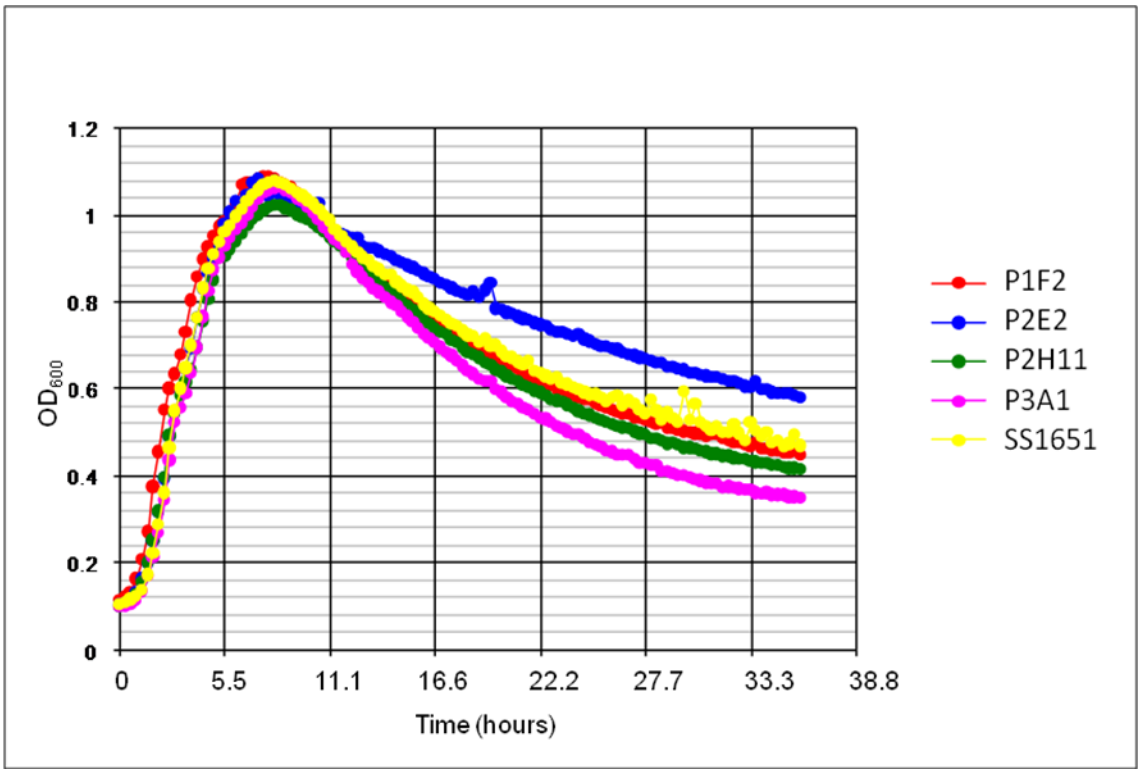


Figure 3.7 Growth curves of 11 TOD$^-$ (B). Cells were grown in 96 well microtiter plates at 37°C with shaking and OD_{600} was measured in a BioTek Synergy HT microplate reader. Growth curves were repeated 3 times.

* SS1651: *P. aeruginosa* NRRL B-23260

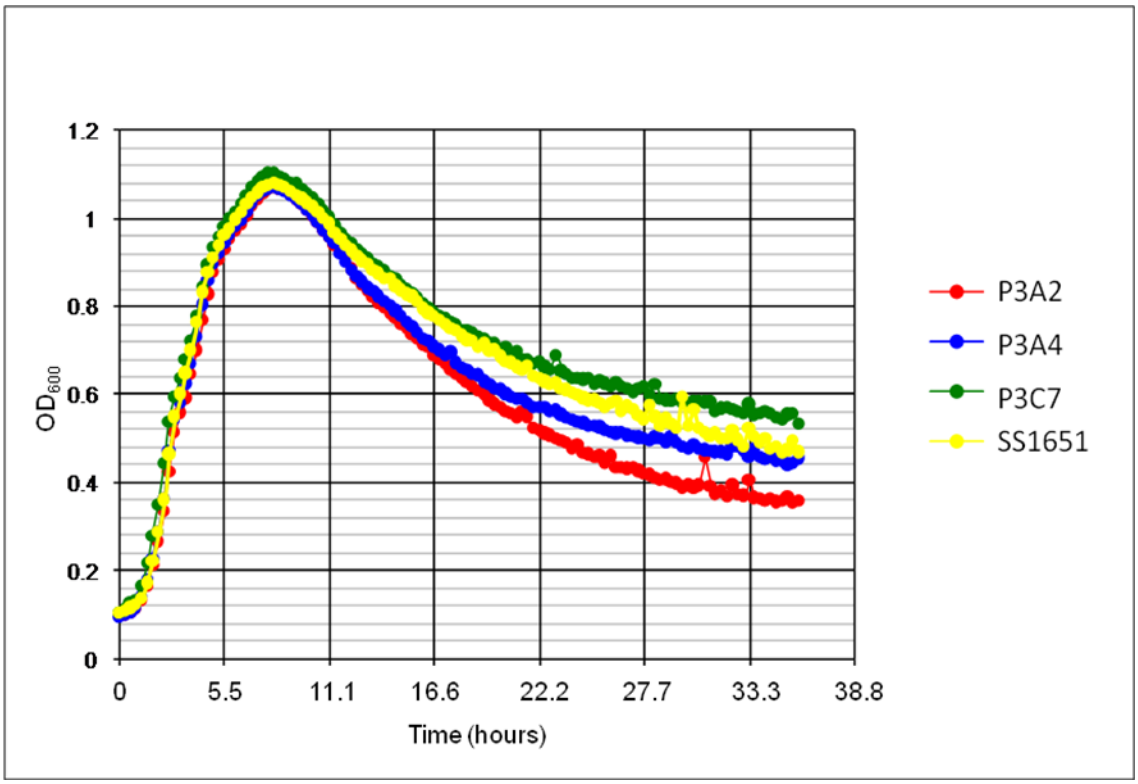


Figure 3.8 Growth curves of 11 TOD$^-$ (C). Cells were grown in 96 well microtiter plates at 37°C with shaking and OD_{600} was measured in a BioTek Synergy HT microplate reader. Growth curves were repeated 3 times.

* SS1651: *P. aeruginosa* NRRL B-23260

sequence obtained was BLAST'd using the *Pseudomonas* Genome Database on www.pseudomonas.com (2007) to identify the gene that was inactivated by transposon insertion (Altschul, *et al*., 1990). Due to the relatively large sizes of the cloned DNA from *Eco*RI digests, we have been unable to determine the identity of the cloned DNA via direct sequencing. Instead, the identity of the affected genes in these mutants was determined via PCR-based mapping (see below). In contrast, the identity of the gene inactivated in the mutant P1E2 was determined to be *PA4358* via direct sequencing of the cloned DNA. *PA4358* encodes for a probable ferrous iron transport protein (FeoB) found in the cytoplasmic membrane.

3.3.1.3. PCR approaches

In addition to the cloning approach, I attempted to identify Tn*5* insertion sites via several PCR approaches: arbitrary PCR, RATE PCR, and TAIL PCR. These approaches have been utilized with varying degrees of success to identify transposon insertions sites in different systems (Karlyshev, *et al*., 2000; O'Toole and Kolter, 1998 (A) and (B); Qian, *et al*., 2005).

The arbitrary PCR approach has been used successfully to identify Tn*5* insertion sites in *P. aeruginosa* and *P. fluorescence* (O'Toole and Kolter, 1998(A) and (B)). The eleven TOD^- mutants discovered from Southern analysis were subjected to arbitrary PCR (O'Toole and Kolter, 1998 (A)) to identify and verify target genes with transposon insertions. In this method, different banding patterns were obtained for each mutant and

DNA bands to be used for sequencing were chosen based on their size and brightness. (Figure 3.9) DNA sequence was determined with Tn*5*Int, and since each mutant gave multiple DNA bands, more than one band was isolated and sequenced to determine the accuracy of arbitrary PCR. Of the multiple bands sequenced for each TOD$^-$ mutant, they all gave the same sequence identification when analyzed by BLAST (Altschul, *et al*., 1990). This demonstrated that arbitrary PCR was amplifying the same flanking sequence but obtaining different sized DNA fragments based on the binding location of the arbitrary primer. Of the eleven TOD$^-$ mutants that were examined using this method, I was able to successfully identify eight insertions sites. The eight identified were P1A1, P1B12, P1E2, P1F2, P2E2, P3A1, P3A2, and P3A4. The interrupted gene in P1A1 was identified to be *PA3306* that encodes for a hypothetical protein that showed homology to alkylated DNA repair proteins in other *P. aeruginosa* strains, as well as *E. coli*. The gene mutated in P1B12 was determined to be *PA5317* that encodes for a probable solute binding protein of an ABC dipeptide transporter located in the periplasm of a cell. P1E2 was mutated in *PA4358* which was the same gene that was identified from direct sequencing of the cloned DNA. These data confirmed that both DNA cloning and arbitrary PCR were accurate approaches to identify the mutated genes. P1F2 was mutated in *PA0914* that encodes for a hypothetical protein with unknown cellular location. Interestingly, upstream of *PA0914* is *PA0913*, which is a probable magnesium transporter in the cytoplasmic membrane. Alignments of the sequence obtained from arbitrary PCR and the wild-type sequence showed that *PA0913* could also possibly be

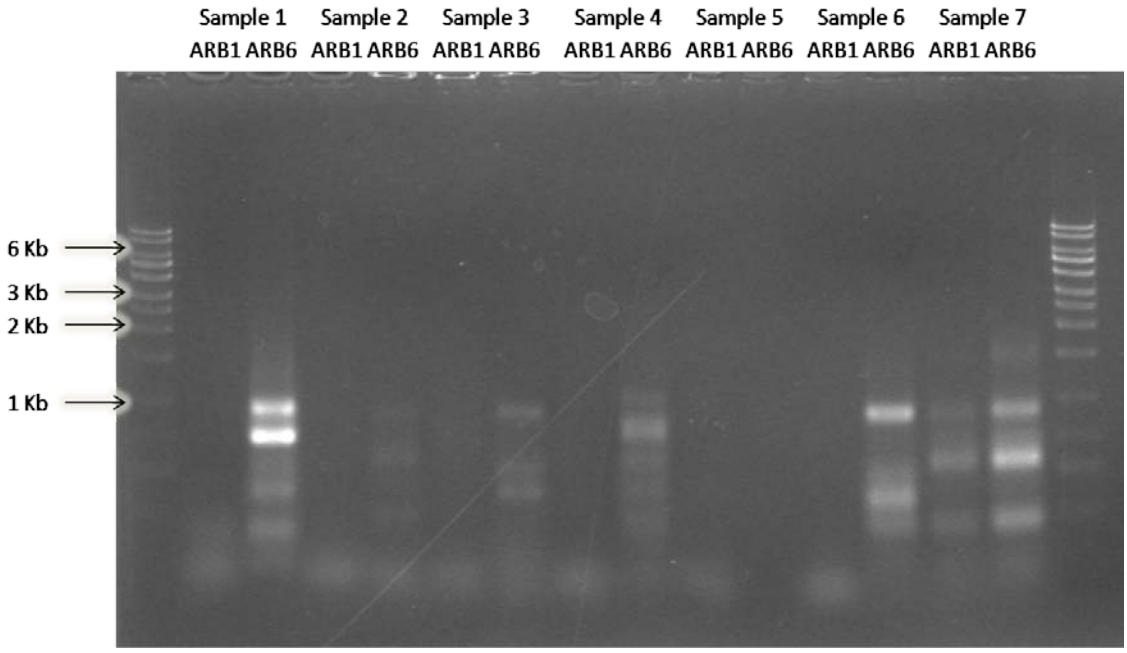


Figure 3.9 Example of arbitrary PCR pattern for TOD$^-$ mutants

Samples 1-7: P1A1, P1B12, P1C2, P1C8, P1D2, P1D5, and P1D9

Each sample was tested with ARB1 and ARB6 to determine the effectiveness of each; as seen in the gel above, ARB6 was the most effective at obtaining PCR fragments

Table 3.4 Identification of Tn*5* insertion sites (*Pseudomonas* Genome Database, 2007)

Putative *tod* Genes			
Mutant	***PA #***	**Gene name**	**Function**
P1A1	*PA3306*	----------	Hypothetical protein (4)
P1B12	*PA5317*	----------	Probable binding protein component of ABC dipeptide transporter (3)
P1E2	*PA4358*	----------	Probable ferrous iron transport protein (3)
P1F2	1)*PA0913* 2)*PA0914*	*1) mgtE* 2) ---------	1) Probable magnesium transporter (3) 2) Hypothetical protein (4)
P2E2	*PA3104*	*xcpP*	Secretion protein XcpP (1)
P3A1, P3A2, P3A4	*PA0733*	----------	Probable pseudouridylate synthase (3)

affected by the Tn*5* insertion in P1F2. P2E2 was mutated in *PA3104* (XcpP) that encodes for a cytoplasmic membrane protein involved in Type II protein secretion system in *P. aeruginosa*. P3A1, P3A2, and P3A4 were all mutated in different regions of *PA0733* that encodes for a probable pseudouridylate synthase found in cytoplasm. These were determined to be genetic siblings, and the transposon inserted into a "hot spot" within the genome. A summary of the mutated genes and their corresponding mutant *tod* mutant designation are shown in Table 3.4.

RATE PCR had been utilized to successfully map the transposon insertions in Karlyshev, *et al*. (2000). I used RATE PCR as an alternative to map my TOD^- transposon mutants. Using RATE PCR, I succeeded in verifying the identity of two of the eight TOD^- mutant targets (P1A1 and P1F2) I had determined with arbitrary PCR (Table 3.4).

The TAIL PCR (Qian, *et al*., 2005) method was unsuccessful in providing unique banding patterns for my transposon mutants when compared to the parent strain (Qian, *et al*., 2005).

3.3.1.4. Verification of Tn*5* insertion sites

Identified transposon insertion sites in various TOD^- mutants were confirmed via PCR analysis. Oligonucleotide primers were designed for each of the identified genes and the PCR amplification profiles were compared to the parent strain NRRL B-23260. In

this analysis, only the parent strain should result in amplification of the gene since insertion of a large transposon (approximately 5 Kb) should disrupt the sequence and would require a much longer extension time to amplify the resulting DNA. As predicted, I was able to amplify the genes of interest only from the parent strain. Although this is negative data, nonetheless, the data support that TOD$^-$ mutants contain transposon insertion in the genes of interest and that is why the wild-type size DNA fragments could not be amplified via PCR (Figure 3.10). In addition, the mutants were tested via PCR using Tn*5*Ext and gene specific primers, based on orientation of the transposon in the gene, to verify transposon insertion in the identified genes. In this analysis, I was able to amplify appropriately sized fragments only from the TOD$^-$ mutants but none from the parent strain (Figure 3.11). Together, these data support and confirm the identity of the genes that are affected in my TOD$^-$ mutants via insertion of the transposon.

3.3.1.5. Genetic complementation of TOD$^-$ mutants

In order to verify the function of the identified putative *tod* genes in the production of TOD, each gene of interest was PCR amplified from *P. aeruginosa* NRRL B-23260 (parent strain) using the *Pfu* DNA polymerase with proof reading function (Stratagene, La Jolla, CA) to minimize spontaneous mutations. The amplified DNA fragments were digested with appropriate restriction enzymes, and cloned into pUCP19, a pUC19 derivative that can replicate in *P. aeruginosa* (West, *et al*., 1994). Thus far, I have cloned the wild-type copies of genes mutated in three TOD$^-$ mutants and introduced them into their corresponding Tn*5* insertion mutants to test for genetic complementation.

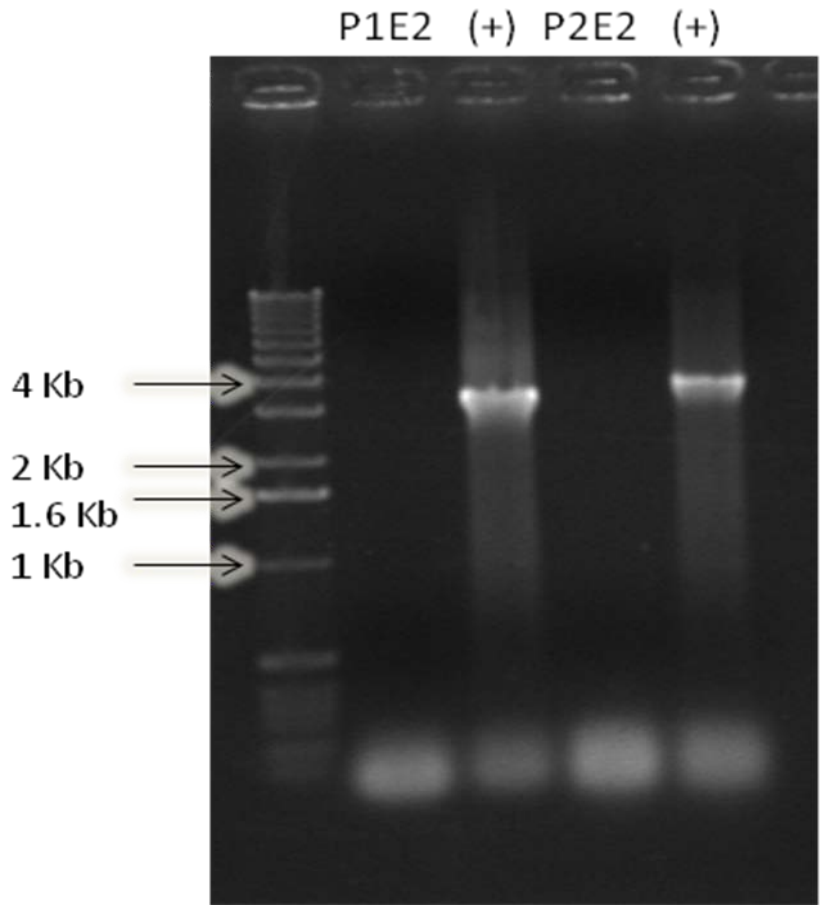
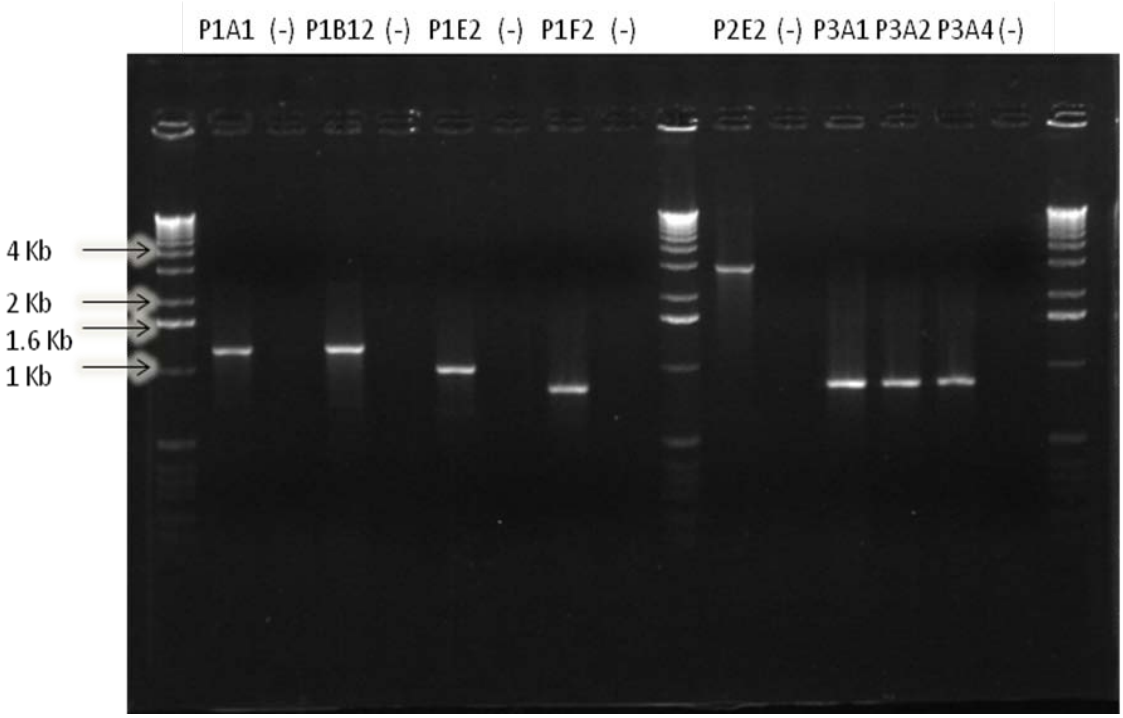


Figure 3.10 Example of PCR verification of TOD^{-} mutants ((+) control is NRRL B-23260)



3.11 Example of PCR verification of TOD$^-$ mutants ((-) control is NRRL B-23260)

These are P1A1 (*PA3306*::Tn*5*-B21), P1E2 (*PA4358*::Tn*5*-B21), P3A1, P3A2 and P3A4 (*PA0733*::Tn*5*-B21). Three of the mutants have mutations in the same gene as shown in Table 3.4. The genetic complementation was initially assessed by visual observation. *P. aeruginosa* strains that convert RA to TOD look strikingly different from TOD^- mutants as demonstrated in Figure 3.12. The TOD^+ *P. aeruginosa* cultures produce milky white supernatants while TOD^- culture supernatants contain clumped cells and globules of lipids. This analysis confirmed genetic complementation of three TOD^- mutants.

3.3.1.6. Testing *PA1169* in TOD production

PA1169 is the only gene in the annotated *P. aeruginosa* genome that is believed to encode for a lipoxygenase. I addressed whether *PA1169* was involved in TOD production. *PA1169* was PCR amplified from *P. aeruginosa* and a null mutation within an open reading frame of *PA1169* was constructed by insertion of a gentamicin resistance cassette, *aacCI* gene (Schweizer, 1993) (Figure 3.13). To introduce the null allele into *P. aeruginosa* via conjugation, I cloned an *oriT* into the plasmid. Following conjugation, *PA1169* knockout mutant of NRRL B-23260 that had undergone allelic exchange was isolated as gentamicin resistant but carbenicillin sensitive strain. I tested the *PA1169* "knockout" mutant to assess whether *PA1169*, a possible lipoxygenase, was involved in TOD biosynthesis. The *PA1169*::*aacCI* "knockout" mutant was tested by fungal assay and by GC analysis. In the fungal assay, the *PA1169* mutant produced supernatant that contained TOD and inhibited *M. grisea* growth. In the GC analysis, the *PA1169* mutant produced as much or more TOD as the parent strain (Table 3.5). Thus, the data

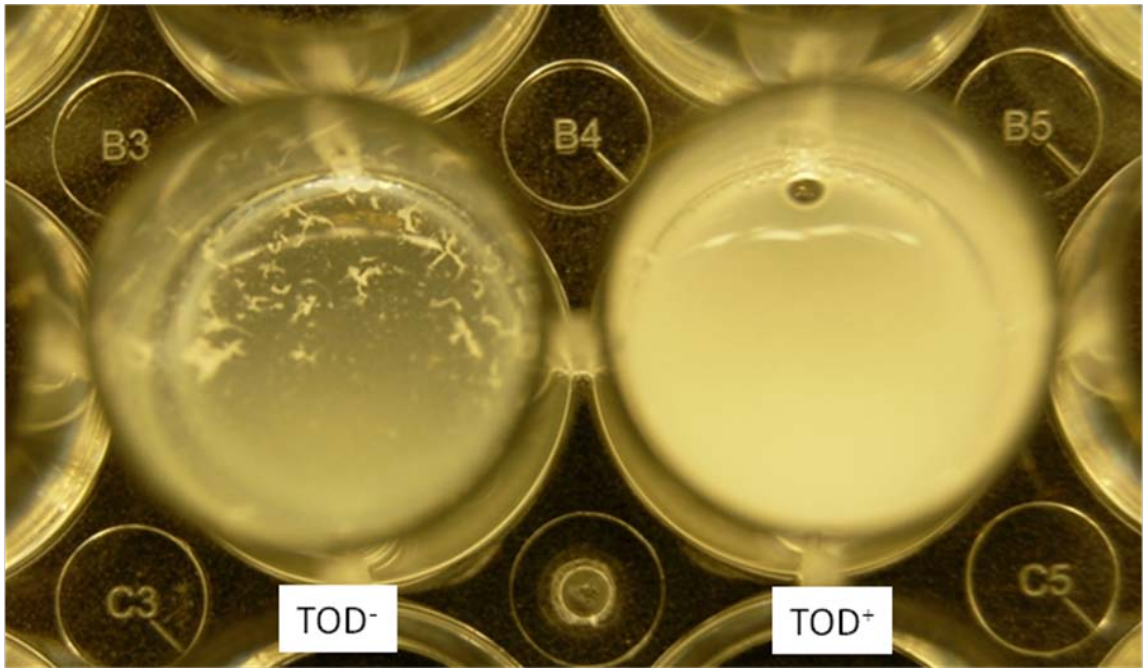


Figure 3.12 Visual observations of TOD^- and TOD^+ cultures. TOD production is characterized by milky, white supernatant and small fat globules as seen with NRRL B-23260 supernatant.

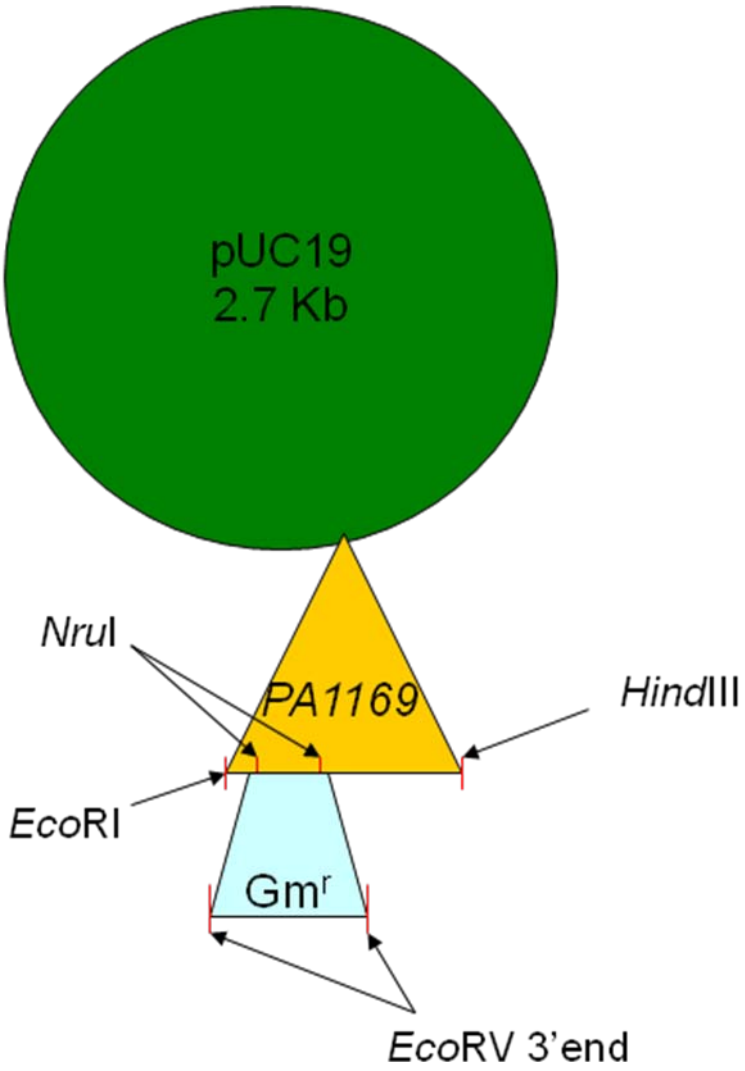


Figure 3.13 *PA1169* "knockout" mutant construction scheme

Table 3.5 Gas chromatography data of *PA1169* null mutants

	Retention time	Response*
NRRL B-23260	17.914	89498
NRRL B-800	17.910	4535
PA1169 clone	17.915	99009

*Response: relative amount of TOD produced

demonstrate that *PA1169* does not encode for a lipoxygenase that is involved in TOD production.

3.4. Discussion

Pseudomonas aeruginosa, a common soil bacterium, possesses the ability to catalyze the conversion of ricinoleic acid into a novel trihydroxy fatty acid, 7, 10, 12-trihydroxy-8(*E*)-octadecenoic acid (TOD). TOD has a potent antifungal activity against important crop pathogens, especially against *Magnaporthe grisea* which causes rice blast disease (Kuo and Nakamura, 2004). In order to elucidate the TOD production pathway in *P. aeruginosa*, I isolated transposon insertion mutants that can no longer convert ricinoleic acid to TOD. In this study, a genetic study was undertaken to map the transposon insertion sites in TOD^- mutants to identify mutated genes. Although I expected to find genes that encode for enzymes such as monooxygenase or lipoxygenase, based on the hypothetical pathway of TOD conversion, I did not find transposon insertions in these specific genes. However, my mutant analysis indicates that TOD production is more complicated than initially believed, and it involves multiple gene products that had not been previously associated with hydroxylation of fatty acids.

I identified several transposon insertion sites using a variety of approaches including several PCR mapping methods and classical cloning of the transposon. As demonstrated in the Results, PCR mapping and cloning complemented each other and confirmed the validity of the data. In addition, when three of the TOD^- mutants (P1A1

(*PA3306*::Tn*5*-B21), P1E2 (*PA4358*::Tn*5*-B21), P3A1, P3A2, and P3A4 (*PA0733*::Tn*5*-B21) were complemented with the wild-type gene in *trans*, observable TOD^+ phenotype was restored. This visual phenotype needs to be verified by the antifungal bioassay. However, the two phenotypes have always been complementary of each other, which suggests the data is valid. Thus, I am confident in my data. Future studies include reconstruction of each mutant with more stable mutant allele such as deletion alleles. This is because Tn*5*-B21 still carries the transposase gene and therefore has the potential for secondary and/or tertiary transposition event.

The identities of several genes involved in TOD production are of interest. *PA3306* (P1A1) encodes a hypothetical protein with homology to alkylated DNA repair protein AlkB. The alkylated DNA repair system repairs DNA that is damaged by alkylating agents (Falnes and Rognes, 2003; Sedgewick *et al*., 2007). Alkylating agents react with the nitrogen or oxygen atoms of DNA to introduce a radical group to the DNA structure that interferes with DNA and/or RNA polymerases (Falnes and Rognes, 2003; Sedgewick, *et al*., 2007). It is believed that the microbial conversion of ricinoleic acid to TOD requires oxygen (Kuo, *et al*. 2001). During aerobic growth, *P. aeruginosa* may generate oxygen radicals which could bind DNA and interfere with the RNA polymerase mediated transcription of the genes involved in TOD production. This inhibition could be relieved by AlkB. Alternatively, the effect of AlkB may be even more indirect. In *E. coli*, AlkB is a part of the adaptive response that is involved in cell survival under oxidative stress conditions (Sedgewick, *et al*., 2007). It is possible that addition of 1%

ricinoleic acid to the stationary phase cells causes stress to *P. aeruginosa* to activate AlkB and that AlkB facilitates detoxification of RA to TOD via unknown mechanism. In summary, how *PA3306* affects TOD production remains to be elucidated.

PA4358 (P1E2) has homology to a possible ferrous iron uptake protein, FeoB. Iron is an essential element and many bacteria require it for growth and survival (Litwin and Calderwood, 1993; Vasil and Ochsner, 1999). Iron is used as a biocatalyst for the function of enzymes, transport of oxygen, and electron transport processes (Litwin and Calderwood, 1993). Since lipoxygenases are iron containing enzymes (Vick and Zimmerman, 1987), a mutation in iron transport would inhibit its enzymatic activity and hence TOD production based on our hypothetical model of TOD conversion.

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PA0733 (P3A1, P3A2, and P3A4) was matched to a probable pseudouridylate synthase which catalyzes post-transcriptional modification of tRNA (Pan, *et al*., 2007). This enzyme is present in most cells in minute quantities and distinct synthases will modify specific RNA sites (Kammen, *et al*., 1988; Pan, *et al*., 2007). Thus, *PA0733* gene product may have indirect effect on TOD production by affecting specific tRNA. As with *PA3306*, the molecular mechanisms by which the product of *PA0733* affects TOD production wait to be elucidated.

I was unable to clone several of the targets into pUCP19 (*PA5317, PA0914*, and *PA3104*), due to unknown reasons. Genes that form secondary structures or that exhibit

potential toxicity at high copy numbers present difficulties for an *E. coli* host. Therefore, they were cloned into pSS213, which is a plasmid vector with a regulatable promoter to prevent gene expression in the absence of induction (Suh, *et al*., 2004). The clones were converted into plasmids that can replicate in *P. aeruginosa* by cloning a mini-stabilizing fragment (Suh, *et al*., 2004). They were introduced into the respective TOD^- mutants: *PA5317*::Tn*5*-B21, *PA0914*::Tn*5*-B21, and *PA3104*::Tn*5*-B21. *PA5317* shows homology to a solute binding protein of an ABC (ATP binding cassette) transporter. ABC transporter is an ATP-dependent solute transport system that consists of two transmembrane domains, two conserved nucleotide binding domains (Binet, *et al*., 1997; Chen, *et al*., 2003), and a solute binding protein. It is likely that this system is involved in the transport of either the TOD biosynthetic enzymes or the long-chain fatty acids across the cellular membranes. *PA0914* is a hypothetical protein that is located directly downstream of *PA0913* which encodes a probable magnesium transporter, MgtE. Based on the location of the transposon, it is possible that *PA0913* is also affected in this mutant. Magnesium transporters are important because Mg^{2+} is involved in numerous cellular functions including as a cofactor for many enzymes (Moncrief and Maguire, 1999; Kehres and Maguire, 2002). Thus, a decrease in cellular concentration of Mg^{2+} may inactivate enzyme(s) involved in TOD production. Finally, *PA3104* encodes for XcpP, a component of the type II secretion system. XcpP secretes proteins across the cell membrane into periplasm where the protein is then taken up by XcpQ to be transported across the outer membrane (Bleves, *et al*., 1999; Filloux, *et al*., 1998; Robert, *et al*., 2005). In 2004, Vance *et al.* demonstrated that in an *xcpP* mutant, although lipoxygenase

was found in the perplasm, there was an 84% reduction in the enzyme activity in the supernatant. This suggested that the periplasm was the natural location of the lipoxygenase. Thus, it is not surprising that a transposon insertion in *xcpP* resulted in a TOD^- mutant phenotype. Future studies include genetic complementation of these mutants. I have introduced the wild-type alleles in *trans* into each of these mutants, which should be tested for TOD production via antifungal assay and GC analysis.

I was disappointed that TAIL PCR failed to amplify an identifiable DNA fragment which could be used to identify transposon insertion sites. I was also surprised that *PA1169,* which is annotated as a putative lipoxygenase in *P. aeruginosa*, did not appear to be involved in TOD production. This suggests that there are other lipoxygenases in *P. aeruginosa* that have not yet been identified. Finally, none of the mutants I mapped thus far have been in genes that had been predicted to be involved in TOD production. This suggests that TOD production is more complex than initially predicted. Future studies should focus on mapping the other 17 TOD^- mutants which I did not pursue. The major reason for mapping these 17 mutants was because the Southern blot data were ambiguous regarding whether these mutants had single transposon insertions. It is possible that these 17 mutants gave ambiguous Southern data due to partially digested genomic DNA. Thus, mapping transposon insertions in these mutants may identify genes for lipoxygenase or monooxygenase involved in TOD production. Alternatively, I may have missed some mutants during the genetic screen or the transposon I used (a Tn*5*) derivative may have not have transposed as randomly as I

had hoped. We have some evidence of non-random insertions since three of eleven mutants I characterized all mapped to the same gene, *PA0733*, which may encode a pseudouridylate synthase. It is also possible that TOD is produced by enzymes whose normal cellular functions are essential. In such a case, a chemical mutagenesis can be performed to look for conditional mutants that are affected in TOD production.

3.5. Summary

Out of twenty-eight TOD$^-$ mutants identified in Chapter 2, I mapped transposon insertions in eleven mutants and determined that eight genes were interrupted. In addition, I successfully complemented three mutants with the wild-type alleles in *trans*. Of the genes identified thus far, none of these appeared to encode for a lipoxygenase or a monooxygenase. These data suggest that TOD production is more complicated than previously thought and involves at least eight genes that have been identified in this study. In addition, I demonstrated that *PA1169*, which had been annotated to encode for a lipoxygenase, is not involved in TOD production. This suggests the existence of an uncharacterized lipoxygenase for TOD production in *P. aeruginosa*.

CHAPTER 4

CONCLUSIONS

The purpose of this research was to identify genes involved in the microbial conversion of ricinoleic acid (RA) to the novel antifungal agent 7, 10, 12-trihydroxy-8(*E*)-octadecenoic acid (TOD). The results from this study were to be the initial steps towards construction of a microbial factory that overproduces TOD via genetic and metabolic engineering of *Pseudomonas aeruginosa*. Such a bacterium can facilitate introduction of TOD to agricultural industry at a large scale to protect rice plants from *Magnaporthe grisea*.

To identify *P. aeruginosa* genes involved in TOD production, I accomplished two major goals. One I was a part of the team (along with Dr. Katheryn Lawrence of the Department of Plant Pathology at Auburn University and her student Taylor B. Hatchett) that developed a high throughput bioassay to test for the antifungal activity of TOD. We also determined the biological range of TOD against various microbial organisms including fungi and soil bacteria (Hatchett, *et al*., 2009). In addition, I isolated transposon insertion mutants of *P. aeruginosa* that were defective for TOD production. I mapped the site of transposon insertion in eleven out of twenty-eight TOD^- mutants to

determine the genes that are involved in TOD production. Initial data suggest that TOD production in *P. aeruginosa* is more complicated than expected and involves at least eight genes.

The development of a high throughput bioassay was essential in order to facilitate the genetic screening of potential TOD$^-$ mutants of *P. aeruginosa*. Initially we tried to develop a plate based assay but found that it was too labor intensive and the throughput assay did not suit our needs. Thus, we developed a 96-well microtiter plate based assay that did not require special equipments other than multiple channel pipettors and a temperature controlled shaking air incubator. This assay is cost effective, simple, and reproducible. Using this assay, we were able to screen 15,168 transposon insertion mutants and identify 28 mutants that were defective for TOD production.

In order to identify *P. aeruginosa* genes involved in TOD production, I took a genetic approach to isolate transposon insertion mutants that were defective in this process. Utilizing Tn*5*-B21, I isolated 15,168 random transposon insertion mutants in *P. aeruginosa* strain NRRL B-23260. This was one of the largest transposon insertion pools to be constructed in *P. aeruginosa*. We isolated over 15,000 mutants because *P. aeruginosa* has over 5800 genes and the goal was to make certain that we had a complete coverage of the genome. Each transposon mutant was tested individually in the bioassay multiple times and from these genetic screens, we identified 28 mutants that were defective for TOD production. Southern blot analysis suggested that 11 of 28 mutants

definitely had single transposon insertions and, thus, I chose to map the transposon insertion sites in these mutants. Transposon insertion sites were identified using several different methods, i.e. arbitrary PCR, RATE PCR, and direct cloning of the transposon. Of the methods, I was most successful with arbitrary PCR which identified eight insertion sites. As demonstrated in Chapter 3 and in Table 3.4, none of the predicted genes were identified in this initial mapping of transposon mutants. However, the identities of the mutated genes suggest several things about TOD conversion. One, ricinoleic acid may require a specific ABC transporter to be imported into the cell before it is secreted into the periplasm. Two, enzyme(s) involved in TOD production may be secreted via the type II secretion system. Three, ricinoleic acid appears to be toxic and TOD production from ricinoleic acid may be a mechanism of detoxification by *P. aeruginosa*. Four, tRNA modification appears to be involved in TOD production.

In summary, the identities of the genes described in this study suggest that further studies are needed to elucidate the molecular mechanisms by which ricinoleic acid is converted to TOD in *P. aeruginosa*. Future studies should focus on completing the genetic mapping of the other 17 TOD$^-$ mutants to identify more genes involved in TOD production. In addition, all of the transposon mutants should be reconstructed with more stable mutant alleles such as deletions. Although we have not observed secondary transposition of Tn*5*-B21, it is still possible because the transposon still carries the transposase gene. Furthermore, genetic complementation of all TOD$^-$ mutants should be completed. I have successfully complemented three out of eight mutants I characterized.

It is important to finish genetic complementation to confirm that the genes are correctly identified. Finally, once the TOD biosynthetic and regulatory genes have been identified, then the strain construction for overproduction can be initiated.

TOD's highly specific antifungal activity against *M. grisea* makes this an ideal fungicide to combat the most widely spread rice pathogen. In addition, because TOD is relatively easily degraded biologically (*P. aeruginosa* consumes it not long after production) (Kuo, *et al.*, 2001), it is ecologically friendlier than synthetic fungicides that are currently in use. Thus, construction of a microbial factory that can facilitate overproduction and isolation of TOD is imperative to make this economically feasible to combat *M. grisea* to save over 150 million tons of rice every year. The study described is the initial step towards achieving this goal.

Future Directions.

The identification of these six TOD biosynthetic genes plays an important role in understanding the mechanism for the production of TOD in *P. aeruginosa*. However, there is a need to test the remaining 17 TOD^- mutants isolated from the fungal assay. These 17 would undergo additional Southern blot analysis to determine if there were double transposon insertions or if previous results were due to incomplete digestion of the genomic DNA. If single transposon insertions are discovered, then the insertion sites would be identified by cloning or PCR methods. It is within these 17 that, hopefully, a possible lipoxygenase or monooxygenase would be identified. All of the genes identified

would be characterized and constructed into an artificial operon under an inducible promoter, which would allow us to "turn on" the TOD biosynthetic genes for probable increased amounts of TOD. Generating a *P. aeruginosa* strain with the ability to produce TOD at an efficiency rate of more than the predicted 45% would give us the means to provide this biological control agent to the public. TOD would be added exogenously to rice plants or perennial rye grasses as a preventative measure against *M. grisea* fungal disease. However, this might present a problem, since TOD has shown to be not only be an effective agent against *M. grisea* but also an effective anti-bacterial agent. This method could interrupt the normal soil flora and cause adverse effects to the plant growth and survival.

As discussed previously, we know that plants produce hydroxylated fatty acids as self-defense molecules in response to fungal infection. We hypothesize that there is also the possibility of introducing the artificial operon into the rice plant with the hopes that it could be activated preventatively as part of the plant self-defense mechanism. Introducing the TOD biosynthetic genes into rice would still require the addition of ricinoleic acid to activate the biosynthetic pathway. Since ricinoleic acid occurs naturally in castor beans and has not shown significant anti-microbial activity, adding it to the rice plants directly would protect the plant from fungal infection and not cause interference in the normal soil flora. I hypothesize this to be the most effective method of control of *M. grisea*.

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