DISSERTATION

INTERACTIONS BETWEEN PLANTS AND AN OPPORTUNISTIC HUMAN PATHOGEN, *PSEUDOMONAS AERUGINOSA*

Submitted by

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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY TIFFANY WEIR ENTITLED "INTERACTIONS BETWEEN PLANTS AND THE OPPORTUNISTIC HUMAN PATHOGEN, *PSEUDOMONAS AERUGINOSA*" BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

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ABSTRACT OF DISSERTATION

INTERACTIONS BETWEEN PLANTS AND AN OPPORTUNISTIC HUMAN PATHOGEN, *PSEUDOMONAS AERUGINOSA*

Pseudomonas aeruginosa is an opportunistic human pathogen that can be found living in soil, water, or saprophytically on plant tissues. It is important to understand the pathology of this organism under variable conditions because of its ability to survive in diverse environments, its role in human disease, and its use as a model organism in studies on biofilm formation, quorum-sensing, and pathogenicity. To this end, a number of unconventional model systems, including plants, nematodes, and fruit flies, have been developed to study the pathology of *P. aeruginosa.* In the present study, the interactions between *P. aeruginosa* and plants, with respect to pathogenicity, quorum-sensing, and microbial ecology are further explored.

To examine what factors are important in the pathogenicity of *P. aeruginosa* in a plant system, compatible and incompatible cultivars of *Nicotiana tabacum* were infiltrated with the pathogen. Bacterial growth *in planta* was monitored and *P. aeruginosa* PAO1 gene expression was examined 24 hours after infiltration into the hosts. The data suggests that, in addition to known virulence factors, the acquisition of micronutrients such as sulfate and inorganic phosphate are also important in disease development. The results of this study also suggest that type III secretion systems may be

important in *P. aeruginosa's* ability to infect plants, and that differences in host response, ie. salicylic acid signaling, are determining factors in host compatibility.

Another aspect of this study was to utilize the natural interactions between plant roots and soil-borne bacteria to identify root exudates that interfere with bacterial quorum sensing (QS), particularly in *P. aeruginosa*. Quorum sensing in *P. aeruginosa* controls the expression of several secreted factors that are important in virulence of the pathogen, and preventing infections by inhibition of quorum sensing is a current therapeutic target. Unfortunately, while many of the exudates appeared to have some affect on QS in general, none had strong activity against *P. aeruginosa* QS systems. However, one class of chemicals, triterpene saponins, was shown to be active in a *lux*-based QS reporter.

Finally, preliminary data suggesting that root exudates can influence competitive outcomes between two soil-borne bacterial species are also presented. The interactions between bacteria are typically studied in nutrient rich medium under defined laboratory conditions. Under these conditions, *P. aeruginosa* outcompetes *Agrobacterium tumefaciens*, two bacteria that potentially compete for the same niche in the soil. However, when *Arabidopsis thaliana* is factored into this equation, growth of *A. tumefaciens* is favored. Furthermore, the negative effects of *P. aeruginosa* on the growth of *A. thaliana* were reduced.

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DEDICTION

I dedicate this work to my son; J. Sebastian "Kiwi" Vivanco that he will always know the endless possibilities that life has to offer.

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CHAPTER 1

Interactions Between *Pseudomonas aeruginosa* and Plants

ABSTRACT

***Pseudomonas aeruginosa*, is a ubiquitous bacterium that can opportunistically infect nearly any compromised tissue and is adept at survival under varying environmental conditions. For these reasons, and because infection by *P. aeruginosa* often results in serious complications for cystic fibrosis patients, it has been the subject of intense study. Here we review studies that have revealed that *P. aeruginosa* employs a similar subset of virulence determinants to elicit disease in animals, invertebrates and plants. Therefore, the results of plant infection studies are relevant to animal pathogenesis. This discovery has resulted in the development of convenient, cost-effective, and reliable plant infection models to study the molecular basis of infection by pathogens such as *P. aeruginosa*. Plant infection models provide a number of advantages in the study of animal pathogenesis. Using a plant model, mutations in *P. aeruginosa* can easily be screened for putative virulence factors, a process, which if done using existing animal infection models, would be time-consuming and tedious. Examining the relationship between *P. aeruginosa* and plants also provides the potential for unraveling the mechanisms by which plants resist animal pathogenic bacteria, offers a means for discovery of novel therapeutic agents, and may shed light on how interactions with plants effect rhizosphere colonization and competitive interactions involving this soil dwelling-bacteria.**

INTRODUCTION

Pseudomonas aeruginosa is an aerobic, Gram-negative, rod-shaped bacterium that, along with the *Burkholderia* and *Xanthomonas* spp., belongs to the Pseudomonadaceae family. These pseudomonads, as they are commonly referred to, inhabit soil, water, and biotic surfaces and are among the few bacterial groups that can be considered true plant pathogens (Starr, 1981). While *P. aeruginosa* is generally saprophytic, it has been shown to infect plants (Elrod and Braun, 1942; Burkholder, 1950; Rahme et al., 1995; Walker et al., 2004) and is the epitome of an opportunistic pathogen in humans. While rarely infecting healthy tissues, it will infect nearly any tissue that has been compromised in some manner. *Pseudomonas aeruginosa* is primarily a nosocomial pathogen that is most commonly found in cystic fibrosis (CF) patients, burn victims, and other immunocompromised patients, as well as soft contact lens wearers and prosthesis users (Fick, 1993). In human pathogenic infections, *P. aeruginosa* has proven to be recalcitrant, as it is resistant to high doses of antibiotics such as ciprofloxacin (Poole, 2000). To date, although chronic *P. aeruginosa* infections in the lungs of CF patients may temporarily respond to antibiotic treatment, in the long run they remain incurable.

One of the contributing factors to its antibiotic resistance is that as a soil-dwelling bacteria, *P. aeruginosa* is frequently in contact with bacilli, actinomycetes, and molds, coevolving resistance to their naturally produced antibiotics (Rahme et al. 1995). However, the primary mechanism responsible for acquired and intrinsic drug resistance in *P. aeruginosa* is thought to be synergy between an impermeable outer membrane and active efflux from the cell (Nikaido, 2003; Li and Nikaido, 2004). In addition, *P.*

aeruginosa maintains transferable antibiotic resistant plasmids (Stewart, 2002) and often colonizes surfaces in its more impervious biofilm form (Singh et al., 2000). Biofilm bacteria are notoriously antibiotic resistant and the molecular basis for this observation is slowly being unraveled (Costerton et al., 1999; Drenkard and Ausubel, 2002; Singh et al., 2002; Stewart, 2002; Mah et al., 2003).

VIRULENCE DETERMINANTS IN INFECTION

Aside from its antibiotic resistant properties, *P. aeruginosa* is such a successful opportunistic pathogen because of its multifactorial pathogenesis. These bacteria possess a vast array of virulence determinants, and infections are both invasive and toxinogenic. Infections of *P. aeruginosa* occur in three steps; each of which is mediated by particular virulence factors. The first step of any *P. aeruginosa* infection is bacterial attachment followed by colonization. Alginate slimes made up of repeating polymers of mannuronic and glucuronic acid provide the matrix for *Pseudomonas* biofilms, which aid in attachment and adherence to host tissues and provide protection against host defenses (O'Toole et al., 2000; Stoodley et al., 2002). Fimbrial adherence is required for respiratory tract colonization by *P. aeruginosa*. One virulence factor, the protease enzyme, may aid in exposing underlying fimbrial receptors on the surface of epithelial cells by degrading fibronectin (Woods et al., 1983). Finally, it is also possible that a surface-bound exoenzyme S aids adhesion of glycolipids to respiratory cells (Baker et al., 1991).

The next step in a *P. aeruginosa* infection, typically occurring in acute infections, is a local invasion that involves the production of myriad toxins and extracellular enzymes that rupture physical obstacles, damage host cells, and provide resistance to host immune responses. One of these extracellular proteases is elastase, which cleaves collagen, IgG, IgA, and complement, as well as lysing fibronectin, disrupting the respiratory epithelium, and interfering with ciliary function (Azghani et al., 2000). Another of these extracellular virulence factors is alkaline protease, which lyses fibrin and prevents new fibrin from being produced (Shibuya et al., 1991). Two other soluble proteins that contribute to local invasion of *P. aeruginosa* are hemolysins, one is a phospholipase and the other is a lecithinase, which synergistically break down lipids and lecithin. Finally, a major determinant in the virulence of the bacteria is the blue pigment, pyocyanin. Pyocyanin impairs the normal function of human nasal cilia (Kanthakumar et al., 1993), disrupts the respiratory epithelium (Lau et al., 2004), and exerts a proinflammatory effect on phagocytes (Ras et al., 1990).

The final step in *P. aeruginosa* infection is toxinogenesis, mediated by two extracellular protein toxins, exoenzyme S and exotoxin A. Both have ADP-ribosylating activity, although exotoxin A is the better characterized of the two toxins (Iglewski et al., 1978). It is identical to the diphtheria toxin in mode of action, but has a different host cell receptor and is antigenically distinct. Its role in both local and systemic disease has been demonstrated by the fact that the purified toxin is lethal (Callahan, 1974; Gray et al., 1984), and that in many cases, *tox-* mutants show attenuated virulence (Rahme et al., 1995).

PSEUDOMONAS AERUGINOSA-PLANT MODEL STUDIES

Aside from being an effective and deadly opportunistic human pathogen, *P. aeruginosa* has been shown to infect plants, animals, and lower eukaryotes (Rahme et al., 2000). In fact, the ability of *P. aeruginosa* to infect multiple hosts led to the development of several non-mammalian models to study its pathogenicity. The first of these models to be developed was a *P. aeruginosa*/plant infection model (Rahme et al., 1995). There were two important observations that led to the use of plant models to study the mechanisms underlying *P. aeruginosa* pathogenesis. First, certain clinical isolates of *P. aeruginosa* had been shown to cause disease in plants (Elrod and Braun, 1942; Burkholder, 1950); and also, it was discovered that Type III secretion systems, which participate in the export of virulence factors, were conserved between human and plant pathogens (Van Gijsegem et al., 1993). This prompted the speculation that common virulence factors were involved in plant and animal pathogenesis. Rahme et al. (1995) were the first to demonstrate that PA14, a clinical isolate of *P. aeruginosa* capable of infecting certain ecotypes of *Arabidopsis thaliana,* used common virulence factors in both animal and plant infections. They showed that mutations in genes encoding two known animal virulence factors, *plcS* and *toxA*, and *gacA*, a gene utilized by *P. syringae*, *P. viridiflava*, and *P. marginalis* in plant pathogenicity, reduced the virulence of PA14 in both an *A. thaliana* and a mouse model. They also utilized a plant model to identify new virulence factors in PA14 by screening 2,500 randomly mutagenized clones of PA14 for decreased virulence on lettuce stems (Rahme et al., 1997). Astonishingly, eight of nine mutants that were less pathogenic on lettuce also showed attenuated virulence in animal infection assays. Molecular analysis of these mutants revealed that two of the mutated genes, *gacA*

and *dsbA*, produced known virulence factors (Peeke and Taylor, 1992; Shevchick et al., 1995; Watari et al., 1995); however, seven of these mutations were in genes that had not previously been implicated in pathogenicity. As the screening of such a large number of mutants would be costly and impractical using vertebrates, the plant model provided an efficient and high-throughput screening tool for the identification of these virulence factors.

Plant models have also assisted in the characterization of several other suspected virulence factors in *P. aeruginosa*. The *degP* gene product in *Escherichia coli* is reportedly responsible for degradation of damaged proteins (Straunch and Beckwith, 1988), growth at high temperatures (Straunch et al., 1989), and protection from heat stress (Speiss et al., 1999). Homologues of *degP* have also been shown to play a role in pathogenesis in both animal (Johnson et al., 1991) and plant pathogens (Stevens, 1998). The role of *mucD*, a PA14 homologue of *degP*, was elucidated using multihost pathogenesis models, including *A. thaliana* as a host plant (Yorgey et al., 2001). *Pseudomonas aeruginosa mucD* mutants form colonies with a mucoid phenotype, which has been attributed to the overproduction of alginate (Boucher et al., 1996), a virulence factor associated with chronic infection in cystic fibrosis patients (Govan and Deretic, 1996). Interestingly, a *mucD* mutant producing more alginate showed decreased pathogenicity in *A. thaliana, Caenorhabditis elegans,* and mouse models (Yorgey et al., 2001). To clarify the role of alginate in PA14 pathogenicity, *algD* a gene involved in alginate biosynthesis, was also mutated. The PA14 *algD* mutant did not show reduced virulence in any of the models tested, suggesting that alginate does not play a significant

role in *A. thaliana, C. elegans,* and mouse pathogenicity, and may only be an important virulence factor in chronic infections requiring biofilm formation.

A similar multihost study was used to characterize the *rpoN* gene, which encodes an alternate sigma (σ) factor in *P. aeruginosa*. This σ-factor is implicated in virulence factor regulation in both plant and animal pathogens (Goldberg and Dahnke, 1992; Totten et al., 1990), and was therefore thought to be a required for multihost pathogenesis. However, an *rpoN* insertion mutation elicited disease symptoms similar to wild-type in *A. thaliana* by 7 days post-infection, and only showed significantly less killing in the *C. elegans* model suggesting that *rpoN* does not regulate the expression of any genes that are universally required for virulence (Hendrickson et al., 2001).

Another interesting application for the plant model is the characterization of virulence factors found in the nontypeable, less virulent strains of *P. aeruginosa* often found in chronic lung infections of cystic fibrosis patients. One of the major constraints in studying pathogenesis of these isolates is that they usually display dramatically attenuated virulence in classic animal infection models. For example, the *P. aeruginosa* strain FRD1 was tested for pathogenicity by transtracheal installation of an agar bead containing 10^4 bacteria in rats (Cash et al., 1979; Woods et al., 1991). FRD1 was unable to establish infection, and the bacterial count was reduced over a period of time. However, the development of a wounded alfalfa (*Medicago sativa*) seedling model was sufficiently sensitive to show disease symptoms using low infectious doses of *P. aeruginosa* strain FRD1 (Silo-Suh et al., 2002). Different virulence factor gene mutations were tested using this model to determine which of these factors may play a role in chronic rather than acute *P. aeruginosa* infections. The genes *rhlR* and *algT* were

found to be important for virulence using the alfalfa seedling model, although mutations in genes encoding other common virulence factors such as *rpoS, pvdS,* and *lasR* did not decrease the virulence of FRD1 on alfalfa. Interestingly, the *algD* mutant, also defective in alginate biosynthesis, did not show reduced virulence in the alfalfa seedling model, agreeing with the previously discussed conclusions of Yorgey et al. (2001) that suggest that alginate is not an important virulence factor in the tested models. Furthermore, an *algT* mutant in a PAO1 background did not show attenuated virulence, suggesting that *algT* controls an unidentified virulence determinant that is important in an attenuated strain like FRD1, but masked in highly virulent strains of the pathogen (Silo-Suh et al., 2002).

QUORUM SENSING

Like many bacterial pathogens, *P. aeruginosa* utilizes a type of molecular crosstalk, or quorum sensing (QS), to control the conditional expression of virulence factor encoding regulons (Latifi et al., 1995). Early studies indicated that this molecular crosstalk in *P. aeruginosa* was mediated by *N*- acyl homoserine lactones (AHLs), or autoinducer molecules, and was dependent on the bacteria reaching a critical density within the host (de Kievit and Iglewski, 2000). Once the critical threshold concentration is reached these molecules begin to bind to QS receptors in the bacteria, which triggers the expression of certain target regulons. However, more recent transcription profiling studies revealed that early addition of AHLs to the culture medium did not influence the timing of expression of many genes thought to be AHL-dependent (Schuster et al., 2003). An alternative hypothesis suggests that there are inhibitors present in culture medium that

must be removed by cellular degradation prior to activation of QS pathways by AHL signal molecules (Yarwood et al., 2005). In addition to inhibitors present in the culture medium, there are a number of environmental factors such as levels of oxygen, iron, and nitrogen that can influence the timing and expression of various QS-regulated genes regardless of AHL levels (Iglewski et al., 1990; Filiatraut et al., 2005).

There are two well-characterized subsystems involved in *P. aeruginosa* quorum-sensing, the *lasR-lasI* and *rhlR-rhlI* subsystems (de Kievit and Iglewski, 2000). The LasR receptor is a transcriptional activator that is thought to be the "master switch" in QS (Gambello and Iglewski, 1991). It is activated by 3-*oxo*-C12-homoserine lactone (Figure 1.1A), generated by the protein product of the *lasI* gene (Pearson et al., 1994). Once LasR is activated, it activates the transcription of a number of virulence factor genes, including the *rhlR* (receptor) and *rhlI* (inducer) genes (Chancey et al., 1999; Latifi et al., 1995). The *rhlI* gene product, an *N*-butyrylhomoserine lactone (Figure 1.1B) generator, binds to RhlR, which leads to the transcription of several more virulence factors (Pearson et al., 1995). Sequence analysis of the *P. aeruginosa* genome revealed a homolog to *lasR* and *rhlR*, named *qscR* (Quorum-sensing control repressor), which lacks a cognate AHL synthase gene (Chugani et al., 2001). Recent studies suggest that QscR responds to the 3-*oxo*-C12-HSL autoinducer and affects the transcription of many genes regulated by the LasR-LasI and RhlR-RhlI QS systems (Lequette et al., 2006). The characterization of these QS regulatory systems does not preclude the possibility of the existence of other major regulatory subsystems. In fact, another QS pheromone, 2-heptyl-3-hydroxy-4-quinolone (Figure 1.1C), named the *Pseudomonas* Quinolone Signal (*PQS*) is required for maximal induction of a subset of QS dependent genes, particularly those involved in

iron acquisition and oxidative stress (Pesci et al., 1999; Bredenbruch et al., 2006). Most of these effects can be traced back to an iron-chelating effect of PQS, suggesting that this molecule acts as a modulator between environmental conditions and QS-regulated gene expression (Bredenbruch et al., 2006).

Because traditional antibiotics are largely ineffective against *P. aeruginosa* infections, it is becoming increasingly necessary to find novel types of treatments. New approaches of pharmacological interference, which prevent the development of infection and the horizontal transfer of antibiotic resistance between bacteria, are becoming a focal point of modern research. An alternative to the traditional approach of antibiotic-mediated bacterial killing is to attenuate bacterial virulence, preventing establishment of infection (Hentzer and Givskov, 2003; Rasmussen and Givskov, 2006). Impeding bacterial QS regulatory systems provides a promising target for these anti-infective or anti-pathogenic drugs. By using treatments that focus on QS inhibition, the selective evolutionary pressures applied by traditional antibiotics would be relieved, but the target bacteria would be rendered non-pathogenic. In addition, there are several possible QS regulatory targets including, AHL signal generation, AHL signal dissemination, AHL signal reception, and constitutive expression of QS repressor genes. Approaching drug discovery with the aim of inhibiting QS has already yielded some promising results. A halogenated furanone derived from those naturally produced by the macroalga, *Delisea pulchra*, was found to inhibit the two QS systems found in *P. aeruginosa* (Manefield et al., 2002; Hentzer et al., 2002, 2003; Hjelmgaard et al., 2003). Furanone treated biofilms showed decreased virulence factor production, without affecting growth or protein synthesis, promoting clearance by the host immune system in a mouse pulmonary

infection model (Hentzer et al., 2002). Furthermore, treatment of *P. aeruginosa* biofilms with QS inhibitors (QSIs) like furanone compounds, garlic extracts, patulin and penicillic acid rendered them more susceptible to the antibiotic tobramycin (Hentzer et al., 2003; Rasmussen et al., 2005a,b). Additionally, there is some evidence to suggest that QSIs aid the activity of polymorphonuclear leukocytes, which use activated oxygen species to kill bacteria, but are unable to develop an oxidative burst in the presence of wild-type *P. aeruginosa* biofilms (Bjarnsholt et al, 2005). Unfortunately, many of the QSIs that have been identified to date are either too toxic to be used in human treatments or would be required in amounts that would likely have severe secondary effects, and there are no QSIs capable of blocking all of the QS-regulated genes (Rasmussen and Givskov, 2006). However, combined treatments using QSIs to render biofilms more susceptible to antibiotics, allowing for improved clearance by host immune systems are still a viable therapeutic possibility.

PLANTS RESPOND TO QUORUM SENSING SIGNALS

An added benefit of using plant models to study human pathogens is that plants are capable of launching chemical responses against infecting bacteria. Therefore, the extracts and root exudates of plants are a potentially rich source of novel QSIs. Discovery and characterization of these plant-secreted compounds could have important biological implications in both agriculture and medicine.

The best-characterized examples of plant-secreted QSIs are halogenated furanones produced by the marine red algae, *Delisea pulchra* (Givskov et al., 1996) (Figure 1.2). These compounds are structurally similar to bacterial AHLs and are capable

of interfering with QS-controlled processes such as swarming and bioluminescence, as well as production of virulence factors and biofilm formation in *P. aeruginosa* (Hentzer et al., 2002). These particular compounds competitively displaced tritiated AHLs from *E. coli* cells engineered to overproduce LuxR receptors (Manefield et al., 1999), leading to reduced LuxR activity by destabilizing the protein, and resulting in accelerated proteolytic degradation (Manefield et al., 2002). Furthermore, halogenated furanone concentrations found on the algal surface were sufficient to prevent Gram-negative bacteria from colonizing algal thalli (Dworjanyn et al., 1999; Kjellberg et al., 1997). QSI activites have also been discovered in extracts of carrot, chamomile, water lily, pepper, chili, and garlic (Rasmussen et al., 2005a), and in several ethnobotanically important plants from south Florida (Adonizio et al., 2006, 2008), although the exact chemical nature of many of these compounds is elusive.

Root exudates in particular offer enticing prospects as a source of QSIs as the rhizosphere, or zone immediately surrounding the roots of a plant, contains a higher proportion of AHL-producing bacteria than bulk soil (Elasri et al., 2001), leading to the speculation that plants can use root-exuded compounds to manipulate bacterial communication. Indeed, a fair body of evidence suggests that cross-talk between plants and bacteria occurs through root-secreted QS signal mimics and/or inhibitors. For example, proteomic analysis of *Medicago truncatula* exposed to bacterial signaling molecules revealed that plants are capable of detecting and responding to these bacterial AHLs (Mathesius et al., 2003). In response to AHLs from *P. aeruginosa* and *Sinorhizobium meliloti,* the accumulation levels of 154 proteins were significantly changed; about two-thirds of these proteins were similar using AHLs from the different

bacteria, but one-third of these were unique to the species of AHL to which the plants were exposed. These data suggest that *M. truncatula* launches a general response to AHLs, but it is also able to distinguish between structurally similar AHLs and accordingly tailor a more specific response. In addition to changes in protein profiles, AHLs also induced specific changes in the chemical profiles of root-secreted secondary metabolites. Several studies have also shown that higher plants, including *Pisum sativum* (pea), *Coronilla varia* (crown vetch), *M. truncatula*, *M. sativa*, *Oryza sativa* (rice), *Glycine max* (soybean), and *Lycopersicum lycopersicon* (tomato), all contain components in their exudates that are capable of stimulating or interfering with bacterial QS in various reporter strains (Teplitski et al., 2000; Keshevan et al., 2005). For instance, swarming in *Serratia liquefaciens* appeared to be specifically induced by *P. sativum* exudates as well as several other plant compounds, as indicated by parallel induction of both swarming and *swrA* gene expression and synthesis of serrawettin, a lipopeptide surfactant required for surface swimming (Eberl et al., 1999). Although QS was stimulated in most cases, the effects of these exudates are sometimes ambiguous. For instance, pea seedling exudates (Teplitski et al., 2000) and a purified AHL mimic from *C. reinhardtii* (Teplitski et al., 2004) resulted in both activation and repression of QS in different reporter strains. These data indicate that QS signal mimics may be widespread in the plant kingdom, and suggest that these compounds interact specifically with different QS receptors or have varying mechanisms of action (Bauer and Mathesius, 2004).

Although QS signal mimics have been found in a range of plant species, they appear to be particularly prevalent among nodulating plants, such as *P. sativum*, *C. varia*, and *M. truncatula*. An intricate two-way signaling between nitrogen fixing rhizobia and

leguminous host plants is required to form a symbiotic relationship. This signaling involves both the secretion of secondary metabolites from plants that stimulate expression of bacterial factors required for nodulation (Peters et al., 1986) and the utilization of QS systems by the rhizobia (González and Keshavan, 2006). Although direct proof remains elusive, indirect lines of evidence suggest that leguminous plants may have evolved the ability to secrete AHL mimics as a means of increasing the efficiency of their nitrogen-fixing symbionts while possibly confusing would-be pathogens by causing them to activate QS-controlled genes before there is a sufficiently large number of bacteria to overcome host defenses.

CONCLUDING REMARKS

Traditionally, plant-microbe studies have focused on the interactions between agriculturally important crop plants and the handful of bacteria and fungi that are capable of either infecting or assisting them. However, the development of cross-kingdom pathogenicity models and the discovery that microbes communicate by diffusible chemical signals has opened up a new realm of possible research avenues. Plants have proven to be inexpensive and efficient yet valuable models for studying human pathogens. A renewed interest in natural products and newly discovered therapeutic targets has revived the search for botanically derived compounds to be used for medicinal purposes. A better understanding of how plants and microbes interact in natural environments is leading to the development of new forms of biological pest control and enhanced methods for rhizoremediation of environmental pollutants (Kuiper et al., 2001, 2004). The following chapters examine how gene expression in the human pathogen, *P.*

aeruginosa is affected in a compatible and incompatible host environment. The root exudates of several plant species from different families is examined for the production of potential QSI compounds. Finally, some preliminary studies examining how plant exudates influence competitive outcomes between the soil-borne bacteria *P. aeruginosa* and *Agrobacterium tumefaciens* are presented.

Figure 1.1: Signaling molecules used for quorum-sensing in *Pseudomonas aeruginosa*. (A) 3-*oxo*-dodecanoyl-acyl-homoserine lactone. (B) butyrl-acyl homoserine lactone. (C) *Pseudomonas* Quinoline signal (PQS).

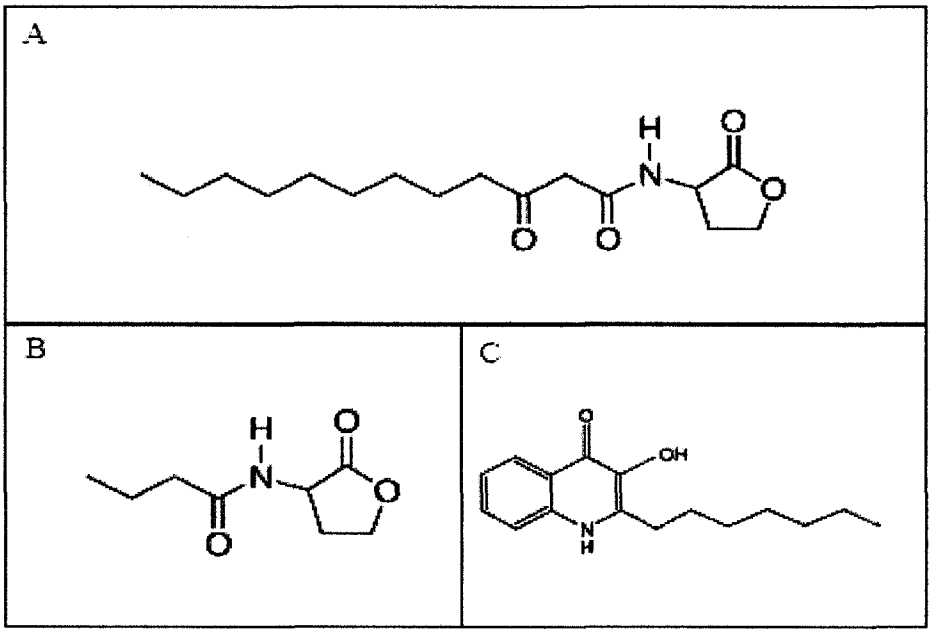
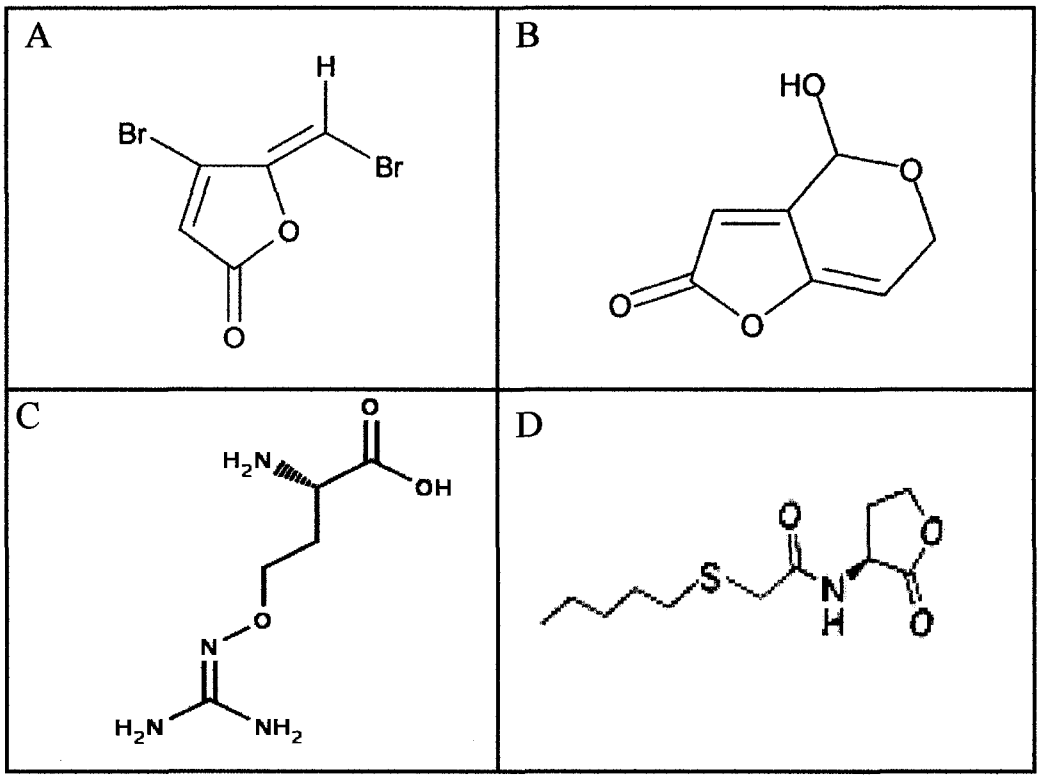


Figure 1.2: Quorum-sensing inhibitor molecules produced by or derived from eukaryotes. (A) Furanone compound 30, based on structures isolated from the marine algae *Delisea pulchra* (Hentzer et al., 2002), (B) Patulin from *Penicillium coprobium* (Rasmussen et al., 2005b) (C) L-canavanine isolated from *Medicago sativa* (Keshevan et al., 2005) (D) A quorum sensing inhibitor derived from garlic extracts (Persson et al., 2005).



CHAPTER 2

Susceptible and Resistant Host Plants Effect *Pseudomonas* Global Gene Expression.

ABSTRACT

Although *Pseudomonas aeruginosa* is an opportunistic pathogen that does not often naturally infect alternate hosts such as plants, the plant-*P. aeruginosa* model has become a widely recognized system for identifying new virulence determinants and studying pathogenesis of this organism. Here we describe how gene expression in *P. aeruginosa* PAO1 is affected *in planta* after infiltration into incompatible and compatible cultivars of *Nicotiana tabacum* L. We determined this information by analyzing global gene expression profiles of bacteria directly removed from the intracellular fluid of the two host plants. We discovered that the availability of micronutrients, particularly sulfate and Pi, are important factors in *in planta* pathogenesis, and that the amounts of these nutrients made available to the bacteria may in turn have an effect on virulence gene expression. Indeed, there are several reports suggesting that *P. aeruginosa* virulence is influenced in mammalian hosts by the availability of iron and by levels of O_2. We also found that the resistant tobacco cultivar had higher basal levels of salicylic acid, and a stronger salicylic acid response upon introduction of PAO1. Salicylic acid acts as a signal to activate defense responses in the plant that could limit the spread of the pathogen and prevent the acquisition of certain nutrients and it has been shown to have direct virulence modulating effects on *P. aeruginosa*.

INTRODUCTION

There are many commonalities between how pathogens of plants and mammals infect their hosts, and between how these hosts recognize and respond to pathogen attack. From the perspective of pathogen infection, the use of type III secretion systems (TTSS) to deliver effector molecules directly into host cells is one of the most notable similarities between plant and animal pathogens (Cornelis and Van Gijsegem, 2000). On the host side, many of the mechanisms that plants and mammals use to identify and respond to these bacterial effectors are also similar (reviewed in Staskawicz et al., 2001, Inohara et al., 2005). For instance, the identification of pathogen-associated molecular patterns (PAMPs) by pathogen recognition receptors (PRR) in the host that activate common signaling pathways and induce core defense responses is conserved (Lee et al., 2006). One type of PRR in plants is exemplified by disease resistance (R) genes whose products recognize pathogen-specific effectors leading to a hypersensitive response that results in the physical isolation of the pathogen. These gene products share molecular motifs, such as ectodomains containing leucine-rich repeats (LRRs) or protein kinase domains, with mammalian Toll-like receptors and nucleotide-binding oligomerization domain (NOD)-containing proteins (Inohara et al., 2005). Another parallel response is the production of reactive oxygen species (ROS) and localized programmed cell death in response to pathogen attack. However, many of the signaling cascades and biosynthetic pathways leading to these responses differ or are only partially conserved (Hammond-Kosack and Parker, 2003; Nurnberger and Scheel, 2001).

In the mid-1990's it was established that plants could serve as useful alternatives to mammalian models for studying pathogenesis of the human opportunistic pathogen

Pseudomonas aeruginosa (Rahme et al., 1995, 1997). These studies revealed that many overlapping factors are required for virulence in mammals and plant infections, which provided the basis for an efficient, high-throughput screen used for identifying new virulence factors. This plant-human pathogen model has been extended to include other human pathogens such as *Enterococcus faecalis* (Jha et al., 2005) and *Staphylococcus aureus* (Prithiviraj et al., 2005b). Another study further characterized the pathogenesis of *P. aeruginosa* in the model plant *Arabidopsis thaliana* and discovered that *P. aeruginosa* congregates on the leaf surface and enters through stomata or wounds. From there it colonizes the intercellular spaces, and can disrupt the cell wall, eventually moving into the vascular system, causing a systemic infection (Plotnikova et al., 2000). In nature, however, *P. aeruginosa* rarely infects plants despite the fact that it is a ubiquitous soil bacterium. Thus, it was reasoned that by examining the global transcriptional response of *P. aeruginosa* in both compatible and incompatible plant models, one could gain insight into what factors are necessary for successful host colonization, whether host defense responses may suppress these factors, and potentially identify new targets for therapeutics.

To date, there have been a number of studies examining host responses to a pathogen at the transcriptome level. For example, the importance of TTSS-mediated suppression of basal defense responses in the compatible plant-pathogen interaction was revealed by examining expression of 7200 *A. thaliana* genes in response to the plant pathogen *P.s syringae* DC3000 and several of its mutants (Thilmony et al., 2006, Hauck et al., 2003). Genes involved in PAMP recognition by *A. thaliana* were identified by examining transcription changes upon infection with DC3000, a TTSS mutant of

DC3000, and a human enteric strain of *Escherichia coli* (Thilmony et al., 2006). Early host responses to *P. aeruginosa* have been examined at the whole genome level in both the fruit fly *Drosophila melanogaster* and in corneal infections of mice (Apidianakis et al., 2005; Haung and Hazlett, 2003).

In recent years, improved molecular techniques have been applied to study the factors that determine successful colonization and infection by various pathogens *in vivo*, particularly *P. aeruginosa*. *In vivo* expression technology (IVET) has proven useful in examining bacterial gene expression in a variety of host tissues and intact hosts, but is limited to examination of induced genes (as opposed to repressed genes), and genes defined to be specifically induced in a particular environment are partially dependent on *in vitro* growth conditions used to assess activation of reporter gene fusions (Rediers et al., 2005). Global transcriptional changes in *P. aeruginosa* have been examined in the presence of human airway epithelial cells and root exudates of *Beta vulgaris* L. (Frisk et al., 2004; Mark et al., 2005). However, there is only one previously published study that has examined global gene expression of this bacteria growing in an intact host. Examination of *P. aeruginosa* from the lungs of a naturally infected patient with cystic fibrosis provided important insights into *in vivo* expression of virulence factors, drug resistance, and nutrient acquisition and utilization (Son et al., 2007). Here, how host factors, basal defense responses, and nutrient availability in a plant host affects *P. aeruginosa* global gene expression 24-hours post-infiltration in two *Nicotiana tabacum* cultivars (cvs. Xanthi nc [NN], Samsun nn) that differ in their susceptibility to infection by this pathogen are assessed. Unraveling the interaction between a plant host and a human pathogen could lead to new insights into how these pathogens behave in a human

host. In addition, determination of why these plant-human pathogen infections rarely occur naturally despite the fact that they are often found in a shared habitat could lead to the discovery of new antimicrobial compounds or other methods for treating diseases caused by these pathogens in humans (Bais et al., 2005). Finally, understanding how human pathogens survive on plants may have implications in food safety practices (Brandl, 2006) as it has recently been recognized that a number of human pathogens can survive on plant tissues both epiphytically and internally (van Baarlen et al., 2007).

MATERIALS AND METHODS

Plant Material and Bacterial Strains

Arabidopsis thaliana Col-0 seeds were obtained from Lehle seed (Roundrock, TX), and *A. thaliana* Ag-O were provided by Dr. Lawrence Rahme. All cultivars, transformants, and mutants of *Nicotiana tabacum* (Samsun nn, Xanthi nc [NN], Petite Havanna SR-1::nn, pTG38, D46H, G216E) were provided by Dr. Barbara Baker (University of California, Berkeley) and propagated in greenhouses at Colorado State University. *Pseudomonas aeruginosa* PAO1 (selected for rifampacin resistance) was generously provided by Dr. E. Peter Greenberg (University of Washington). Cultures were routinely grown in Luria-Bertani (LB) broth in a 37°C rotary shaker at 200 rpm and rifampicin (50 $\mu g\ ml^{-1}$) was used for antibiotic selection for plate counts.

Determination of Bacterial Pathogenesis in *A. thaliana* and *N. tabacum*

Overnight cultures of PAO1 were diluted to $1x10^7$ cells ml^{-1} (for *A. thaliana* infections= $2x10^5$ cells ml^{-1}) in 10mM $MgSO_4$. Five hundred microliters of diluted culture were infiltrated into three leaves on each treated plant (resulting initial inoculum was $5x10^6$ cells $leaf^{-1}$ for *N. tabacum* and $1x10^5$ cells $leaf^{-1}$ for *A. thaliana*). Plants were injected with 500 µl of 10 mM $MgSO_4$ for controls. Plants were incubated in temperature-controlled chambers at 28-30°C and high humidity. Colony forming units (CFUs) were obtained at 24-hour time points for five days by removing infected leaves, washing the surfaces with mild disinfectant, and infiltrating them to saturation with sterile dH_2O. Saturated leaves were placed in cotton-filtered syringes positioned into a centrifuge tube and spun at 5000 rpm for 10 minutes to remove intracellular fluid. Intracellular fluid levels were adjusted and diluted with sterile dH_20 and plated on LB solid medium containing 50 µg ml^{-1} of rifampacin to prevent the growth of other bacterial species present on the leaf tissues. Cultures were incubated at 37°C and colonies were counted after 24 hours. Three leaves from treated and control plants were plated for each time point and all experiments were conducted at least two times. Figures represent average data from all of the experiments and the error bars represent ±1 standard error.

RNA Extraction and Microarray Analysis

Infections and bacterial recovery for microarray analysis were carried out as described in the previous paragraph for *N. tabacum* except leaves were infiltrated to saturation. PAO1 was removed from infected leaves 24 hours after treatment. Bacterial pellets were flash frozen in liquid nitrogen and RNA was isolated using a Ribopure RNA

isolation kit (Ambion Inc., Austin, TX) according to the manufacturer's instructions with a slight modification. Bacterial cells were vortexed 3x for 10 minutes to increase efficiency of mechanical cell lysis, resulting in higher concentrations of recovered RNA. The additional DNAse I treatment was included to minimize contamination by chromosomal DNA. Samples were concentrated using the Rneasy MiniElute Cleanup kit following the manufacturer's instructions (Qiagen Inc., Valencia, CA). The concentration of RNA was assessed using a Nanodrop system (Nanodrop, Wilmington, DE) and RNA integrity was determined by agarose gel electrophoresis and by 28S/18S ratio on an Agilent 2100 bioanalyzer (Agilent, Palo Alto, CA). Preparation of cDNA, labeling, and hybridization to Affymetrix *P. aeruginosa* GeneChips (Affymetrix Inc., USA) were conducted at Asuragen Inc. (Asuragen, Austin, TX) using the following instruments: GeneChip 640 hybridization oven, GeneChip 450 Fluidics Station, and a high-resolution GeneChip 3000 scanner (GeneChip, Santa Clara, CA). Microarray hybridizations were performed in triplicate using RNA/cDNA obtained from concurrently conducted independent experiments. The data analyses were performed with GeneSpring 7.2 (Silicon Genetics, Redwood City, CA) DNAChip Analyzer. The data were normalized per chip by dChip invariant set normalization, and each gene was normalized to the median measurement taken for that gene across all samples. An average two-sample *t*-test using a P value cutoff of ≤ 0.05, a present call in at least two replicates, and an average two-fold or greater change compared to controls was applied to identify genes that were statistically differentially expressed. All gene annotations are from the Pseudomonas Genome Project website.

Synthesis of cDNA for Quantitative PCR

Several genes that were differentially regulated in susceptible host versus resistant host infection were chosen for validation using quantitative real-time PCR. RNA was isolated as described above and cDNA was prepared using the procedure described by Affymetrix (Affymetrix, Santa Clara, CA). Briefly, *P. aeruginosa* RNA was incubated in a thermal cycler with random primers purchased from Invitrogen Life Technologies (Invitrogen, Carlsbad, CA) at 70°C and 25°C for 10 minutes each and cooled to 4°C. Incubated primer/RNA mix was then added to cDNA synthesis components and incubated in a thermal cycler (25°C for 10 min, 37°C for 60 min, 42°C for 60 min, 70°C for 10 min, chill to 4°C). RNA was removed by addition of 1N NaOH and incubated at 65°C for 30 minutes followed by addition of 1N HCl to neutralize the reaction. MiniElute PCR Purification Columns (Qiagen Inc., Valencia, CA) were used to concentrate the samples and cDNA quantity was determined using a Nanodrop system (Nanodrop, Wilmington, DE).

Real-Time Quantitative PCR

To validate the microarray analysis, prepared cDNAs were subjected to quantitative real-time PCR using seven primers for genes that were shown to be up- or down-regulated (Table 2.3). An iCycler IQ5 (Bio-Rad Laboratories, Hercules, CA) real-time PCR machine was used for the quantification of cDNA. For quantitative analysis of gene transcripts by qRT-PCR, PCR reactions were performed using a SYBR GreenER qPCR Supermix for iCycler instruments (Invitrogen Corp, Carlsbad, CA) according to the specifications of the supplier. qRT-PCRs were performed in 25 µl mixtures

containing 21.5 µl of Master Mix, 2.5 µl of cDNA, and 0.5 µM (each) forward and reverse primers. External standard curves to determine primer efficiency were generated using 5 dilutions (10^1-10^5) of genomic DNA extracted from bacteria recovered 24 hours after infiltration into *N. tabacum* cv Samsun plants. The PCRs were performed in triplicate for each gene and sample. Reaction mixtures were amplified using the following cycles: 1 cycle of 50°C for 2 min and 95°C for 10 min 30 s; 40 cycles of 95°C for 15 s and 60°C for 1 min; 1 cycle of 95°C for 1 min and 55°C for 1 min. To correct for differences in the amount of starting material, the ribosomal gene *rpsL* was chosen as a reference gene for normalizaton. Fold change ratios were determined using a variation of the Livak method using the following equations:

$$\text{Relative Expression (E)} = 2^{C_T(\text{reference}) - C_T(\text{target})}$$

$$\text{Fold change ratio (FC)} = \text{E reference} / \text{E target}$$

This modified method was used because the amplification efficiencies of the target and reference gene primers were similar but were not near enough to 100%.

Analysis of Inorganic Phosphate and Sulfate

To determine levels of inorganic phosphate (Pi) and sulfate in the two tobacco cultivars, plants were infected as described above and incubated overnight at 28-30 °C. Approximately 1 g of fresh tissue per repetition was removed and infiltrated with sterile dH_2O and the intracellular fluid was removed by centrifugation as described. Recovered intracellular fluid from each repetition was centrifuged and filtered through a

0.2μ filter to remove bacteria. The resulting fluid was placed in sterile containers and diluted to a total volume of 100 ml with sterile dH_2O and stored in the dark at 4°C until processing. Samples were processed at the Colorado State University's Soil, Water and Plant Testing Laboratory by Dr. James Self. Briefly, sulfate concentrations from intracellular fluid were determined by ion chromatography using a Dionex 2000i/SP (Dionex Corporation, Sunnyvale, CA) and Pi concentrations were determined by a Molybdate blue calorimetric method on a Milton Roy Spectronic-20 (Milton Roy Company, Ivyland, PA). Specific protocols are described in *Standard Methods for the Examination of Water and Wastewater* (American Public Health Association, 1992).

Total Salicylic Acid Content

Total salicylic acid levels were measured for tobacco cultivars (Xanthi and Samsun) using a modified version of protocols described by Bowling et al. (1994), Scott et al. (2004), and Prithiviraj et al. (2005a). Briefly, plants were infiltrated with 500 μl of PAO1 diluted to $1x10^7$ cells ml^{-1} or 10 mM $MgSO_4$ per leaf and incubated overnight at 28-30 °C. One gram of fresh tissue per repetition was removed and stored at -80°C prior to extraction. Tissue was ground in liquid nitrogen and extracted at 5°C in 80% methanol. Samples were sonicated and centrifuged for 20 min at 13,000 rpm in a tabletop centrifuge. Supernatant was removed and the pellet was re-extracted as described above. The two supernatants were combined and evaporated to dryness. The sample was resuspended in 2.5 ml of 5% trichloroacetic acid and sonicated for 10 minutes. Free salicylic acid was separated from conjugated forms by extraction with 2 volumes of ethyl acetate:cyclopentane:isopropanol (50:50:1). The organic phase was evaporated to dryness and resuspended in 0.5 ml of MeOH. The aqueous phase was acidified to pH 1 with 1N

HCl and boiled for 30 minutes to release conjugated salicylic acid. The organic phase was re-extracted as described and resuspended in 0.5 ml MeOH. Samples were analyzed by HPLC on Dionex Acclaim, Polar Advantage II C18 column (5μm, 120Å, 4.6 x 150 mm) and separated using a 10-95% gradient of MeOH:4 mM formic acid over 15 minutes at a flow rate of 0.5 ml min^{-1}. Comparison of peak areas using a salicylic acid standard (Sigma, St. Louis, MO) was used to quantify samples.

RESULTS

PAO1 Infection of *A. thaliana* and *N. tabacum*

To study the host-induced gene expression in *P. aeruginosa*, we first evaluated the virulence of PAO1 in two potential host plant species, *A. thaliana* (ecotypes Col-0 and Ag-0) and *N. tabacum* (cv. Xanthi nc [NN] and Samsun nn). Three leaves on each test plant were infiltrated with PAO1 (*N. tabacum*: $5x10^6$ cells $leaf^{-1}$; *A. thaliana*: $1x10^5$ cells $leaf^{-1}$) and maintained at 28-30°C for the duration of the experiment. The high level of inoculum used in *N. tabacum* was necessary to ensure that a sufficient number of cells could be recovered in subsequent experiments for RNA extraction 24 h post-inoculation. However, differences in pathogenicity between the two tobacco cultivars were seen using initial inoculum levels as low as $2x10^5$ cells $leaf^{-1}$ (data not shown). Plants were monitored visually for symptom development and three leaves from each cultivar/ecotype were removed daily over a period of five days and recovered bacteria were assessed for viability. Infiltration of *A. thaliana* with PAO1 resulted in no significant differences in CFU counts between the two ecotypes (Figure 2.1A), and both ecotypes displayed characteristic necrosis and chlorosis of the infected leaves that progressed to wilt and

resulted in plant mortality. Despite replication of experimental methods, these results are contrary to previously published data showing differential infection of these same ecotypes by *P. aeruginosa* strain PA14 (Rahme et al., 1995, 2000).

Leaf infiltration in the two tobacco cultivars culminated in a successful infection in cv. Samsun, whose symptoms included chlorosis and necrosis in the infected area, which eventually spread to other tissues of the plant. No visible symptoms were observed in cv. Xanthi aside from necrosis around the injection site, likely resulting from mechanical damage during leaf infiltration (Figure 2.2). The CFU counts of bacteria recovered from the intercellular space in the infiltrated tobacco leaves corroborate this phenotypic observation (Figure 2.1B). After five days, the PAO1 population recovered from cv. Xanthi was only slightly higher (~3 fold) than initial inoculum levels, suggesting that it is an incompatible host for these bacteria. Conversely, bacteria recovered from cv. Samsun continued to multiply and resulted in the recovery of approximately 50-fold more cells five days post-inoculation than the initial inoculum levels.

PAO1 Gene Expression Analysis

To examine how cultivar-specific factors affected PAO1 growth and virulence *in planta*, global gene expression was examined in bacteria recovered from the intracellular spaces of the infiltrated leaves of cvs. Xanthi and Samsun. To unravel determinants of early infection, gene expression in *P. aeruginosa* was examined 24 hours post-infiltration because there was less difference in bacterial growth between the two cultivars at this time point, thus minimizing differences in gene expression due to population density

differences. Utilizing Affymetrix GeneChip microarray technology, approximately 7% of the genome was differentially expressed ($P \leq 0.05$; fold change ≥ 2) with genes involved in adaptation and protection, transport, and energy metabolism being some of the largest functional categories affected (Figure 2.3A, Appendix A). Using PAO1 gene expression from *N. tabacum* cv. *Samsun* (susceptible to PAO1 infection) as the calibrator data set, only 1% of the differentially expressed genes were determined to be up-regulated and about 6% were down-regulated (Figure 2.3B).

A large number of the genes that were up-regulated are involved in sulfate metabolism and some have been shown to be overexpressed in *P. aeruginosa* cells grown under conditions of sulfur stress (Quadroni et al., 1999). These up-regulated genes include components of sulfate transporters, genes involved in transport and metabolism of alternative sulfur sources, and a gene thought to encode a protein utilizing selenocysteine (Table 2.1). As expected, there were a number of genes related to virulence that were down-regulated in PAO1 recovered from resistant host plants (Appendix A). Among these genes were *rpoS*, a stationary phase σ–factor that positively regulates the expression of a number of exoproteins, including endotoxin A and alginate (Suh et al., 1999). Not surprisingly, genes involved in the biosynthesis of alginate (*algE, alg44*) and endotoxin A (*toxA*) were also down-regulated. Several genes involved in Type II and Type III secretion systems were also down-regulated. Six of these protein transport genes were probable components of TTSS, including three *psc* genes that are thought to be components of the "injection needle" (Yahr et al., 1996) and *pcrV,* which is involved in effector translocation (Yahr et al., 1997). It was also interesting to note that a number of genes involved in transport and metabolism of phosphorus, particularly

inorganic phosphate (Pi) were also down-regulated (Table 2.2). In addition to being an essential micronutrient for the bacteria, Pi is thought to be important in motility and biofilm formation in *Pseudomonas spp.* (Rashid et al., 2000; Monds et al. 2007). Down-regulation of these genes in the non-host plant suggests that Pi is not a limiting micronutrient in that environment or that plant-specific factors could be modulating the expression of these genes.

qRT-PCR Validation of Microarray Data

Seven genes that were differentially expressed in the microarray analysis were chosen for relative quantification of the transcripts using quantitative real time PCR (qRT-PCR). Primers were designed for three genes that were up-regulated and four genes that were down-regulated (Table 2.3). The ribosomal gene *rpsL* was used to normalize expression of the different treatments and biological replications. All of the genes chosen followed the same trends of up-or down-regulation that were seen in the microarray analysis, although the fold changes varied (Figure 2.4). However, there was a great deal of variation in expression between biological replications. Extracting a sufficient amount of bacterial RNA for these and the microarray experiments required a large number of plants of both cultivars and due to space constraints the sources of RNA used to make the cDNA were isolated from separate experiments that were conducted at different times and from plants that were grown under similar conditions but in different growth chambers. Because of this variation, the standard deviation between the relative expression (E) of the calibrator samples (*N. tabacum* cv. Samsun) and the treatments (*N. tabacum* cv. Xanthi) are not significantly different for many of the genes examined.

Determination of *in planta* Levels of Sulfate and Pi

As discussed, some of the most obvious differences in gene expression between bacteria recovered from compatible versus incompatible plant material were the activation or repression of several genes involved in the uptake and metabolism of sulfate and Pi. To examine the cause of these differences, we mimicked the infiltration and collection protocol used for the microarray experiments to examine the available levels of sulfate and Pi present in the intercellular fluid. Mock-inoculated plants were used as controls. We discovered that the amount of sulfate recovered from the intercellular fluid did not differ significantly between cultivars or treatments (P=0.544) (Figure 2.5A). This suggests that there are no inherent differences in the level of available sulfate between these cultivars that would account for the activation of sulfur stress pathways in PAO1 residing in the intracellular space of cv. Xanthi. However, it does not rule out the possibility that cv. Xanthi is able to physically confine the invading bacteria, creating a microenvironment where sulfate is more limited.

Assessment of Pi levels in the intracellular fluid could be interpreted in two ways. Statistical comparisons using Fisher (LSD) revealed that levels of PO_4 were significantly higher in the intracellular fluid of infiltrated cv. Xanthi than in infected cv. Samsun (Control, P=0.106; Treated, P=0.039), suggesting that the differences in gene expression related to phosphorus uptake and metabolism could be due to the availability of this nutrient in the different plant cultivars (Figure 2.5B). Although comparisons using the more stringent Tukey's test (HSD) suggest that the amount of Pi does not differ in either control or treated plants of these cultivars (Control, P=0.139; Treated, P=0.134)

suggesting that the difference is small compared to the variability between plants and may not be an important factor in determining gene expression related to phosphorus utilization.

N-gene Involvement in Resistance to PAO1

In some cases, resistance of a plant to a specific pathogen is conveyed by the presence of a resistance gene that has evolved to overcome a virulence gene of a specific pathogen. In the case of *N. tabacum*, the presence of a dominant N-gene triggers a hypersensitive response upon infection by tobacco mosaic virus, and results in resistance to the virus (Dinesh-Kumar et al., 1995). Although the "gene for gene" interaction is thought to be a very specific interaction between one host gene and one pathogen gene, there have been reports of a single R gene conferring resistance to multiple attackers (Grant et al., 1995; Rossi et al., 1998). Because one tested tobacco cultivar had a dominant N-gene (cv. Xanthi nc [NN]), and the other had a recessive N-gene (Samsun nn), and the presence of a dominant N-gene correlated with resistance to PAO1, it was necessary to determine if PAO1 resistance was mediated by the N-gene or by other factors that differed between the cultivars. To determine if the source of resistance to PAO1 was N-gene related, *N. tabacum* cv. Petite Havanna SR-1 (SR-1::nn) and a transformant of this cultivar, pTG38, that contains a full length N-gene and shows hypersensitive response to TMV infection (Dinesh-Kumar et al., 1995) were infiltrated with PAO1, as performed in earlier experiments. Two N-gene mutants, D46H and G216E, that were transformed into an SR-1::nn background and crossed with wild-type Samsun NN (Dinesh-Kumar et al., 2000) were also infected. The D46H mutant carries a

point mutation in the toll-interleukin 1 receptor (TIR) and the G216E mutant carries a mutated nucleotide binding site (NBS) region. None of these infiltrations resulted in a successful infection (Figure 2.6A and 2.6B), suggesting that differences between cultivars independent of N-gene expression are responsible for resistance against PAO1.

Salicylic Acid levels in *N. tabacum* Cultivars

Salicylic acid is an important defense signal in N-gene mediated and N-gene independent responses to pathogen attack in tobacco plants. In addition, as little as 0.1 mM of salicylic acid has been shown to have a direct effect on the virulence of *P. aeruginosa* strain PA14, resulting in reduction of several virulence factors including pyocyanin, elastase, and protease, and deterring biofilm formation (Prithiviraj et al., 2005a). In fact, several of the same genes were up- or down-regulated similarly in both studies, although these datasets were obtained using different time points (10 h post-exposure to SA vs 24 h post-infection *in planta*) (Table 2.4). To determine if a stronger salicylic acid response might contribute to the resistance of cv. Xanthi against PAO1, we examined post-infiltration and mock infiltration levels of SA in both Xanthi and Samsun cultivars. The basal levels (mock-inoculated) of total salicylic acid in leaves of cv. Samsun was significantly lower than the levels detected in cv. Xanthi (P=0.021) (Figure 2.7). However, the most significant difference was seen in the response of the two cultivars 24 h after inoculation with PAO1. Levels of total salicylic acid detected in the PAO1-infected cv. Samsun (susceptible to PAO1 infection) were higher than basal levels, but did not differ significantly from the basal levels detected in the Xanthi cultivar (P=0.205) and were approximately 2.5x lower than the amount of total salicylic acid

found in PAO1 infected cv. Xanthi ($P<0.0001$) (Figure 2.7). These data suggest that cv. Xanthi launches a more effective defense response against *P. aeruginosa* and that the amount of salicylic acid identified in the leaves may be sufficient in this cultivar to have direct negative effects on PAO1.

Figure 2.1. (A) A logarithmic graph of PAO1 cells recovered from the leaves of *A. thaliana* ecotypes Col-0 and Ag-0 during a four-day infection period show no difference in the susceptibility of these ecotypes. However, (B) a logarithmic graph of PAO1 colony forming units (CFUs ml^{-1} intracellular fluid) recovered from infiltrated *Nicotiana tabacum* cvs. Xanth nc [NN] (resistant) and Samsun nn (susceptible) tissue over a five-day time period suggests that these cultivars display differential susceptibility to PAO1. Data represent mean values from 3 replicate leaves per time point and each experiment was performed in triplicate. Error bars represent ±1 SE.

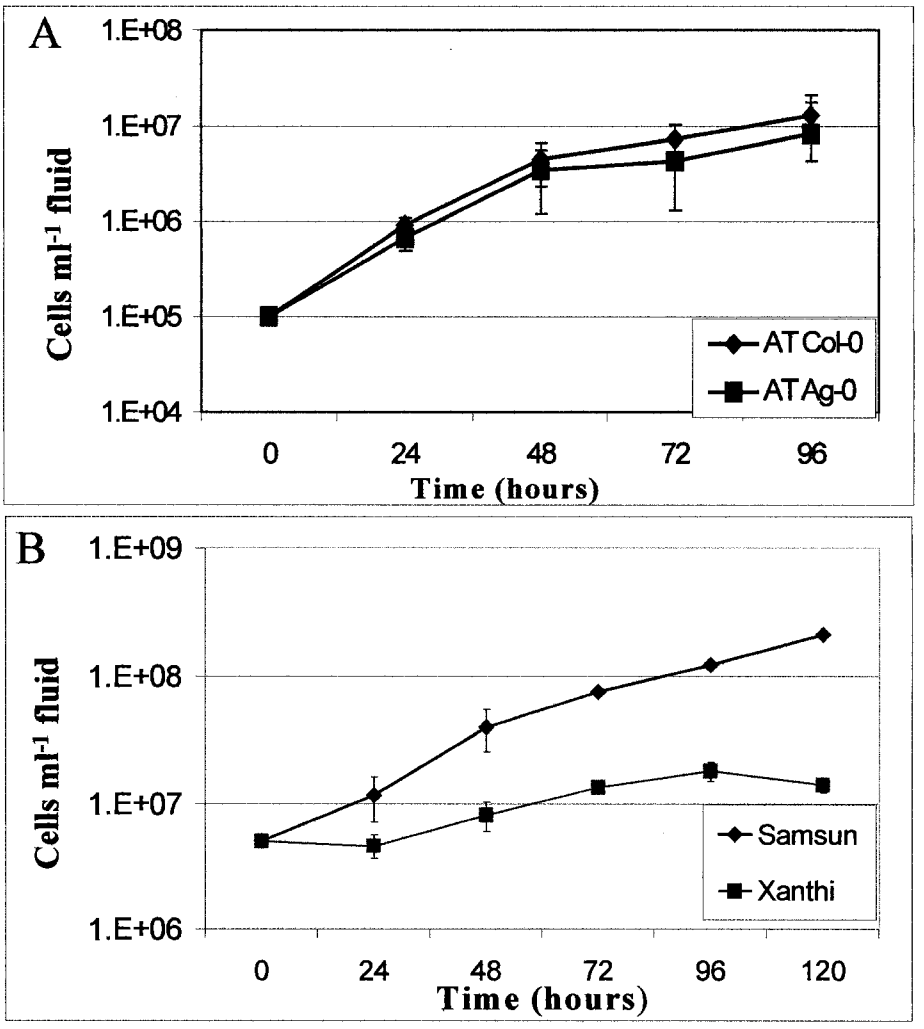


Figure 2.2. Leaves of *N. tabacum* cv. Samsun (susceptible to PAO1) and cv. Xanthi (resistant to PAO1) five days after infiltration with 10mM $MgSO_4$ and PAO1. Arrows indicate infiltrated areas and the circle highlights infected tissue.

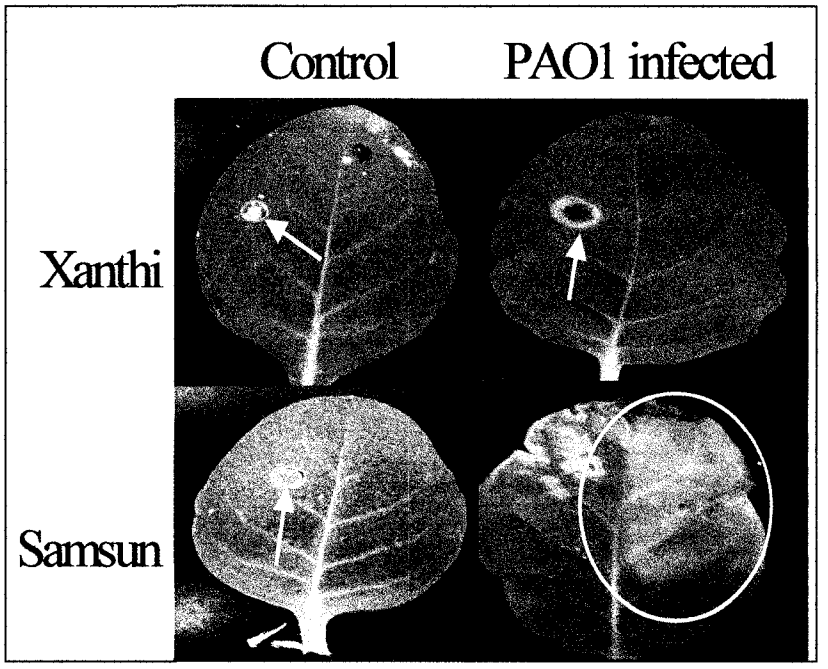


Table 2.1. Genes involved in sulfur uptake and metabolism that were up-regulated in PAO1 from *N. tabacum* cv. Xanthi (non-host plants).

Gene name	Fold Change	p-value	Predicted Function
PA3445	2.36	0.035	conserved hypothetical sulfonate, nitrate, bicarbonate transporter
cysT	2.73	0.010	sulfate transport protein CysT
cysD	3.02	0.040	ATP sulfurylase small subunit
sbp	3.20	0.005	sulfate-binding protein precursor
cysI	3.36	0.019	sulfite reductase (cysteine biosynthesis)
PA3446	3.44	0.007	conserved hypothetical protein (70% similarity to E. coli SSP4)
PA5181	3.61	0.029	probable oxidoreductase (selenocysteine containing protein)
cysN	3.64	0.028	ATP sulfurylase GTP-binding subunit/APS kinase
cysW	3.86	0.023	sulfate transport protein CysW
cysA	4.22	0.002	sulfate transport protein CysA

Figure 2.3. (A) Functional categories of up- or down-regulated genes in *Pseudomonas aeruginosa* reisolated from the leaves of *N. tabacum* cv. Xanthi (resistant to PAO1) relative to bacteria reisolated from cv. Samsun (susceptible to PAO1). (B) Total percentage of genes differentially expressed greater than 2-fold at a significance level of $P<0.05$.

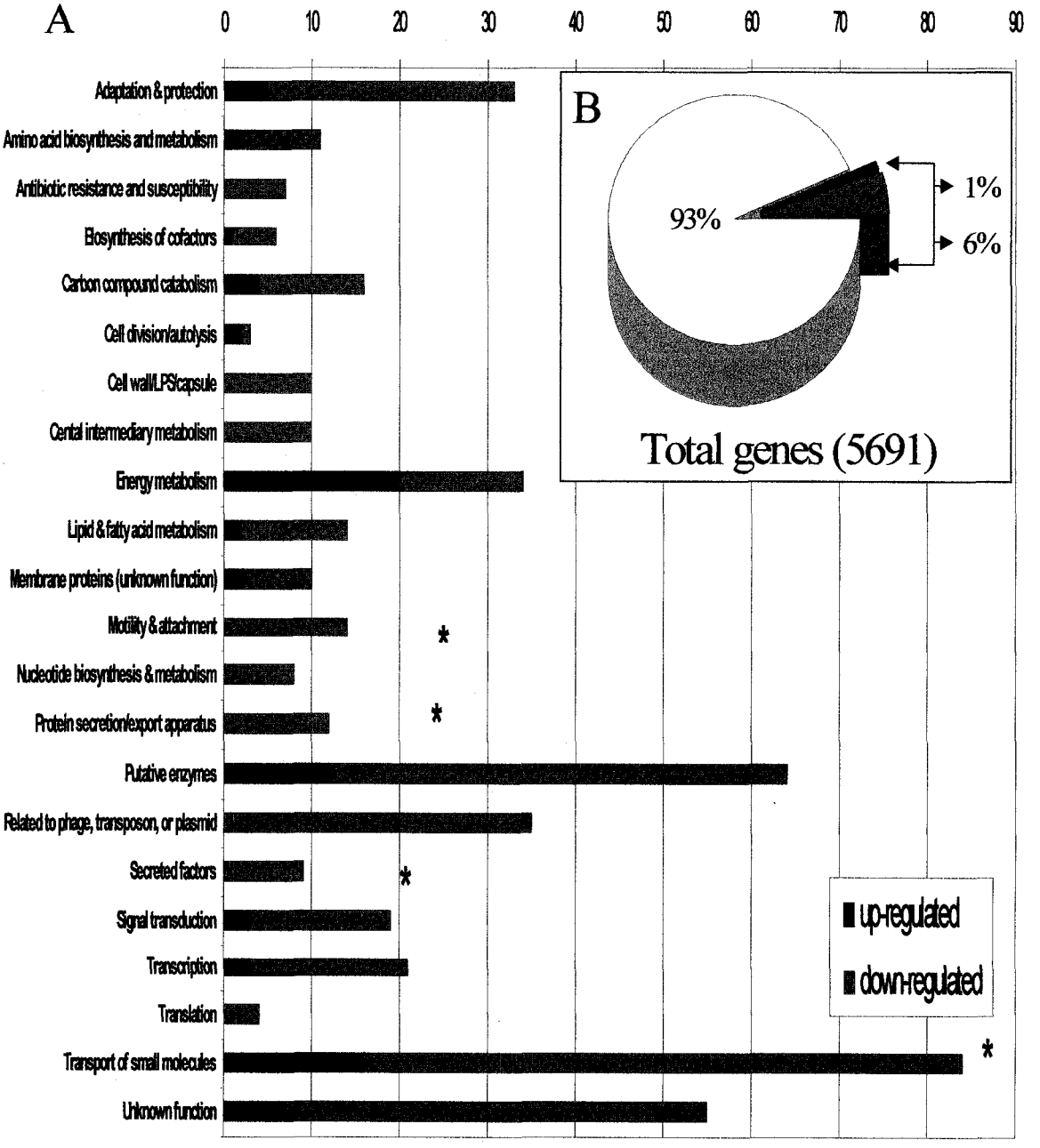


Table 2.2. Genes involved in phosphate uptake and metabolism that were down-regulated in PAO1 from *N. tabacum* cv. Xanthi. While many of these genes are involved in the acquisition of phosphate, several genes (*plcN, katA, phnX*) are also thought to be involved in *P. aeruginosa* virulence.

Gene name	Fold Change	p-value	Predicted Function
phnC	5.17	0.01	ATP-binding component of ABC phosphonate transporter
PA3383	1.74	0.022	binding protein component of ABC phosphonate transporter
PA4861	5.92	0.030	probable ATP-binding component of ABC phosphate transporter
PA3376	5.98	0.043	probable ATP-binding component of ABC phosphate transporter
PA0450	6.03	0.011	probable phosphate transporter
oprP	6.24	0.042	Phosphate-specific outer membrane porin
oprO	7.07	<0.001	Pyrophosphate-specific outer membrane porin
phoA	3.93	0.004	alkaline phosphatase
phoB	2.33	0.009	two-component response regulator
plcN	5.37	0.036	non-hemolytic phospholipase C precursor
glpQ	2.38	0.041	glycerophosphoryl diester phosphodiesterase
katA	2.03	0.034	catalase
pqqc	2.15	0.014	pyrroloquinoline quinone biosynthesis protein C
phnX	2.62	0.002	2-phosphonoacetaldehyde hydrolase

Figure 2.4. Fold changes in gene expression in *P. aeruginosa* reisolated from *N. tabacum* cv. Xanthi (X) relative to those isolated from cv. Samsun. The magnitude of the change differs between this experiment and those obtained from microarray analysis, but the directional trend remains the same for all of the genes tested.

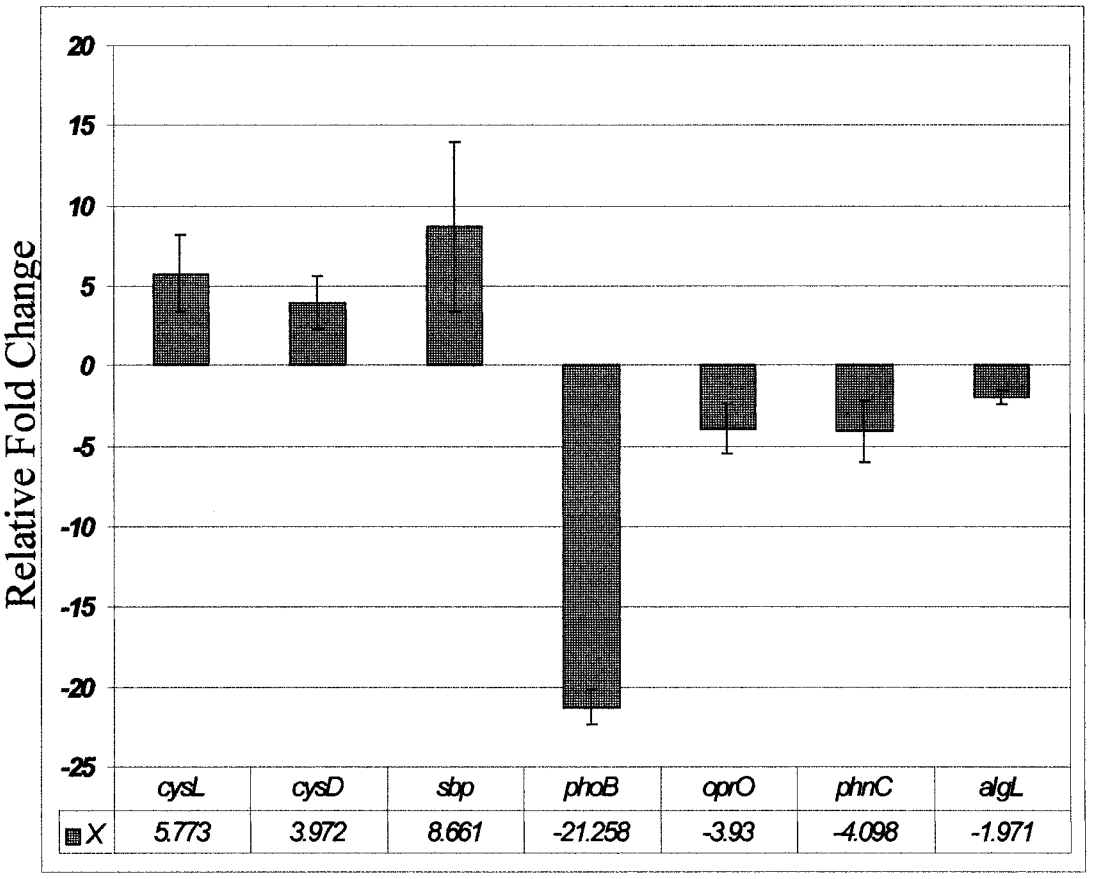


Table 2.3. Sequences of primers used for qRT-PCR of selected differentially regulated genes.

Gene	Name	Primer Sequence
PA1838	*cysI*	F: GTCACCGACAAACAGCTGGA
		R: TCGGCGAGAATGATGTTCTG
PA4443	*cysD*	F: TCTGATCACCCACGTCAACC
		R: TACTTGTCCAGGGCCTGCTT
PA0283	*sbp*	F: CGAGAAGGCCAAGGACTACG
		R: CCGATCTGGTTGTTGACGAA
PA5360	*phoB*	F: GGACCTGATCCTGCTCGACT
		R: GGTCAGTTCGTCACGCTTCA
PA3280	*oprO*	F: ACGCCTACAAGGACATCAAG
		R: CACCTTCCAGCTTGTACTGG
PA3384	*phnC*	F: CTGCATCAGGTCGACTACG
		R: GAAGCCTTCGGAAAGGTACT
PA3547	*algL*	F: AAGTACGAAGGCTCCGATTC
		R: ATGTCGGTGATGTCCTTGAT

Figure 2.5. (A) Amount of sulfate and (B) inorganic phosphate detected in the intercellular fluid of *N. tabacum* cvs. Xanthi and Samsun 24 hours after infiltration with PAO1 or 10 mM $MgSO_4$. Data represent the mean of value of four replicates and error bars represent ±1 SE.

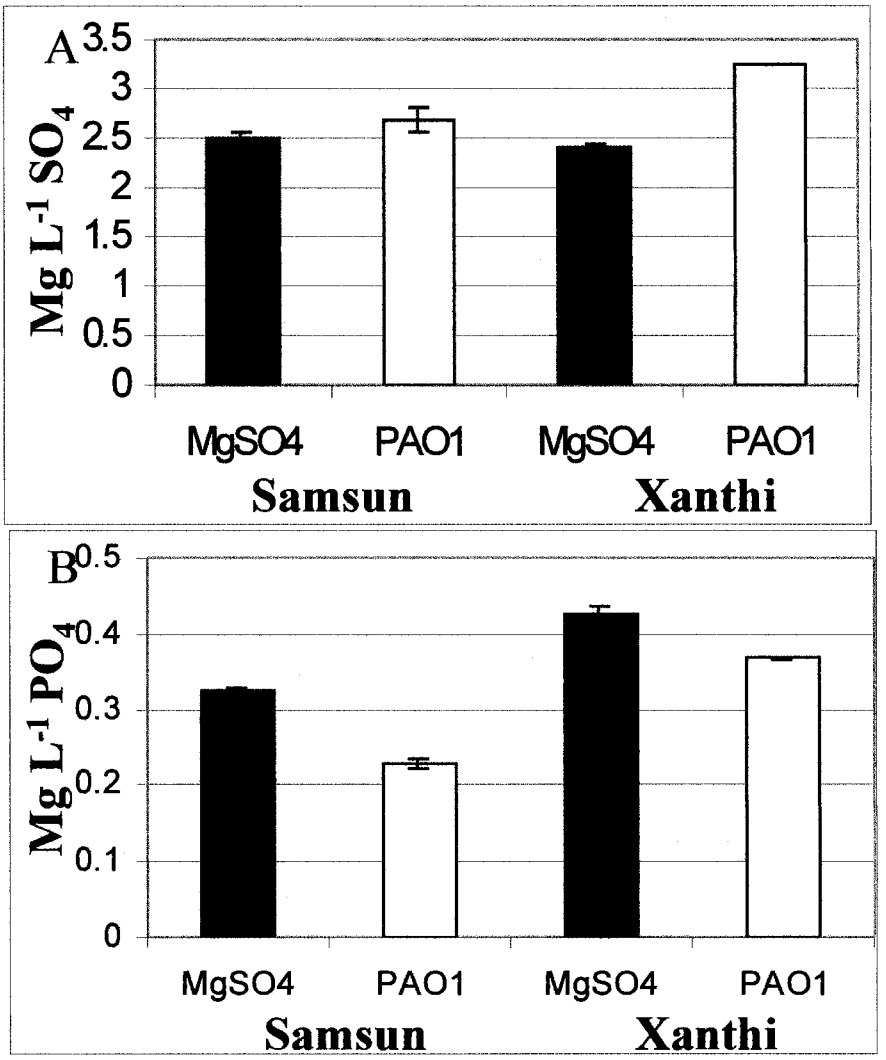
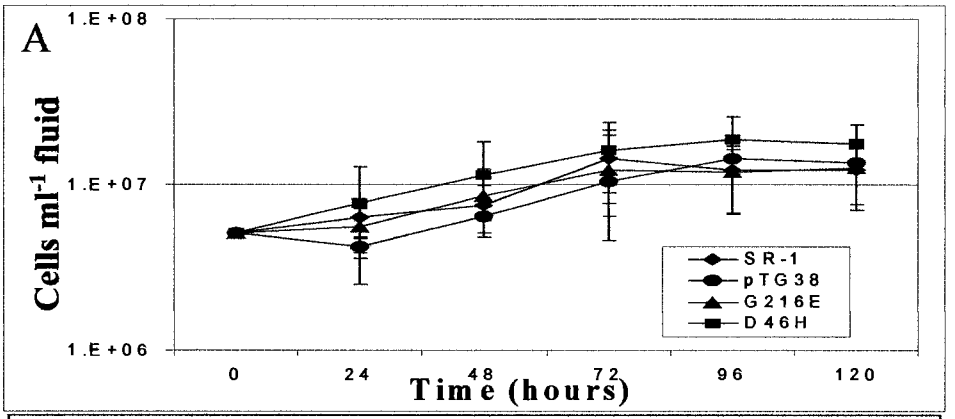


Figure 2.6. (A) The number of PAO1 colony forming units (CFUs) recovered from infiltrated tissue of *N. tabacum* cv. Petite Havana SR-1, which lacks a dominant N-gene did not differ significantly from a transformant carrying the dominant N-gene (pTG38) or two N-gene mutants (G216E, D46H), suggesting that this resistance gene plays no role in preventing infection by *P. aeruginosa*. Data represent mean values from 3 replicates per experiment with each experiment performed at least twice. Error bars represent ±1 SE. (B) Infiltration of PAO1 did not result in symptom development in either *N. tabacum* cv. Petite Havana SR-1 or the N-gene transformant, pTG38. Arrows highlight areas of infiltration.



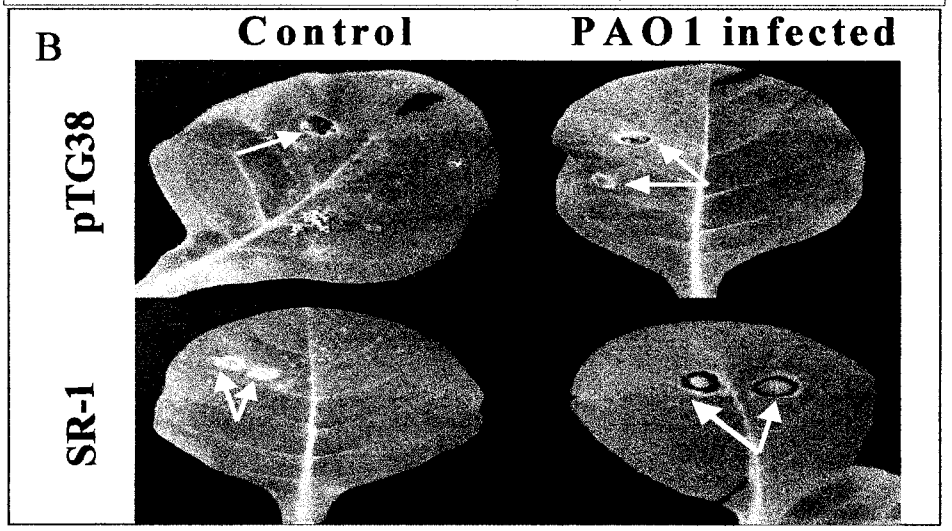
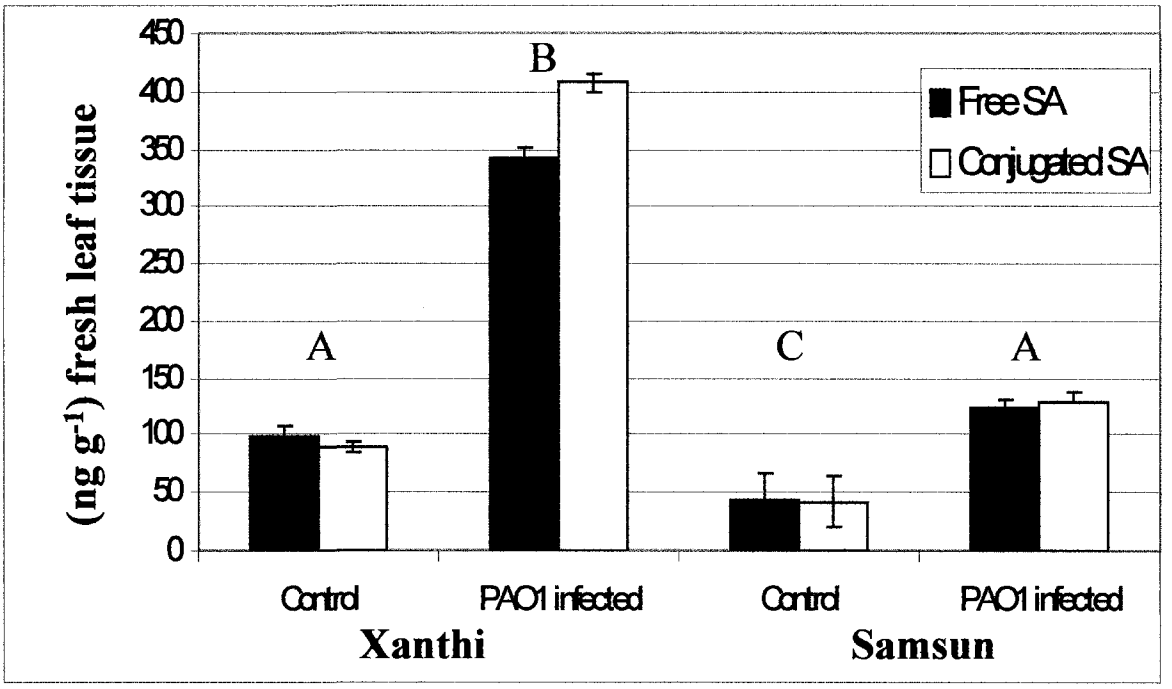


Table 2.4. PAO1 genes similarly regulated in response to both salicylic acid exposure and infection in *N. tabacum* cv. Xanthi. Comparison is from microarray analyses done at 24-hours post infection ([1] this study) and 10 hours after exposure to salicylic acid ([2] Prithiviraj et al., 2005a).

Gene Name	Predicted Function	Fold Change[1]	Fold Change[2]
PA0508	probable acyl-CoA dehydrogenase	3.01	3.08
PA0673	hypothetical protein	-3.34	-2.48
PA0685	probable type II secretion system protein	-4.82	-2.74
PA0776	hypothetical protein	-4.58	-2.96
PA1327	probable protease	-2.46	-2.84
PA1700	conserved hypothetical protein in type III secretion	-2.36	-6.78
pcrV	type III secretion protein PcrV	-2.28	-3.52
PA2263	probable 2-hydroxyacid dehydrogenase	3.07	4.52
PA2575	hypothetical protein	4.08	2.58
PA3188	probable permease of ABC sugar transporter	-7.20	-2.78
glpD	glycerol-3-phosphate dehydrogenase	3.88	11.98
phaC2	poly(3-hydroxyalkanoic acid) synthase 2	-3.04	-3.80

Figure 2.7. Amount of free and conjugated salicylic acid in 1 gram of leaf tissue 24-hours after mock- or PAO1 infiltration into *N. tabacum* cvs. Samsun (susceptible to PAO1 infection) and Xanthi (resistant to PAO1). Data represent mean values of 6 individual replications from duplicated experiments, error bars represent ±1 SE, and different letters denote significantly different means.



DISCUSSION

The study of plant interactions with *P. aeruginosa* has revealed a great deal about individual factors that are necessary for virulence of this bacterium (Rahme et al., 1995, 1997; Yorgey et al., 2001; Silo-Suh et al., 2002). In fact, the success of these studies has validated the use of plant models to examine key processes involved in the establishment of host-pathogen interactions (van Baarlen et al., 2007). However, these studies were conducted using random or specific mutants of *P. aeruginosa* and reveal very little about the holistic interaction between *P. aeruginosa* and the model host. To address this deficiency, comprehensive transcriptional changes in PAO1 in the context of a compatible and incompatible interaction with *N. tabacum* plants were examined.

One of the most obvious differences identified in PAO1 gene expression was the induction of sulfur starvation pathways in bacteria removed from resistant host plant tissue. Sulfur starvation occurs in three primary stages. First, the bacteria upregulate genes involved in acquisition of sulfate, the preferred sulfur source. Subsequently they begin to up-regulate genes for acquiring and utilizing secondary sources of sulfur, and ultimately to synthesize alternative forms of proteins containing reduced amounts of cysteine (Quadroni et al., 1999). We found many genes that are required for sulfate acquisition (*sbp*, genes in *cysTWA* operon) and one gene encoding a selenocysteine-containing protein, were up-regulated in the non-compatible host environment, suggesting early stages of sulfur starvation. The levels of sulfate present in the intracellular fluid of both cultivars of *N. tabacum* did not differ significantly, indicating that only the amount of sulfate available to the bacteria was restricted. Although a visible

hypersensitive reaction was rarely visible on PAO1-infected leaves of cv. Xanthi, the data suggest that the plant is able to effectively create a physical barrier that restricts the growth of the bacteria and prevents them from accessing sulfur and other intercellular nutrients.

Another large group of differentially regulated genes were involved in the transport and metabolism of inorganic phosphate (Pi). Inorganic phosphate is an essential micronutrient required for bacterial growth but it has also been implicated in the regulation of biofilm formation in *Pseudomonas fluorescens* via phosphate-dependent modulation of c-di-GMP levels, which control secretion of a surface adhesion (Monds et al., 2007). Relative to the calibrator dataset (compatible host, cv. Samsun), many genes required for acquisition and metabolism of Pi were down-regulated in non-compatible host plants (cv. Xanthi). These results were contrary to what might be expected if resistant host plants are preventing a flow of nutrients to PAO1. However, Pi is required in a much smaller quantity than sulfate and it is possible that the effects of Pi starvation in resistant host plants would not yet be visible at 24-hours post-infection. This would explain why no up-regulation of Pi acquisition genes was seen, but does not explain why these genes appear to be down-regulated in cv. Xanthi (the resistant host plant). While it is enticing to speculate that defense related factors produced by resistant host plants were able to modulate the expression of these genes to hinder the bacteria from acquiring Pi, an examination of Pi levels in the intercellular fluid offers a much more mundane explanation. Using Fisher's LSD comparison, there was a significantly lower amount of Pi in PAO1-infected samples from *N. tabacum* cv. Samsun than in the infected cv. Xanthi samples ($P=0.039$). Thus, it is possible that the resulting differential expression was due

to the activation of Pi starvation pathways in cv. Samsun as there was somewhat less Pi available in these plants. The up-regulation of *oprO* and *oprB*, which are membrane porins specific for Pi and pyrophosphate support this hypothesis. Additionally, the activation of *Pho* genes that are induced under low Pi conditions and regulate genes such as *phnC* suggest that the bacteria are optimizing their ability to utilize alternative sources of Pi (Wanner and Boline, 1990). However, basal levels of Pi in the two cultivars did not differ significantly (P=0.106), and using the more stringent Tukey's HSD statistical comparison suggests that there was also no significant difference in the amount of available Pi between the two PAO1-infected cultivars (P=0.134). This may indicate that differences in gene expression are not a result of Pi deficiency, but rather that bacteria retrieved from host plants (cv. Samsun) have begun to utilize Pi in pathogenesis-related processes and need to acquire additional Pi beyond the basic requirements for survival. It has been proposed that many Pi regulated genes in *P. aeruginosa* function as a Pi scavenging system that may be critical to pathogenesis (Liu, 1979; Weinberg, 1974). Many of the Pi acquisition and metabolism genes that were up-regulated in bacteria retrieved from host plants relative to their non-host counterparts (*plcN, phoA*, *glpQ* and *oprO*) were also shown to be up-regulated after exposure to human airway epithelial cells (Frisk et al., 2004), and one differentially regulated gene in this study, *plcN*, is a nonhemolytic phospholipase that has been associated with virulence in *P. aeruginosa* (Darby et al., 1999; Konig et al., 1997).

An important virulence determinant of PAO1 in mammalian hosts is the secretion of exotoxins via the TTSS (reviewed in Frank, 1997). The TTSS functions by facilitating the direct injection of bacterial effector proteins directly into host cells

(Staskawitz et al., 2001; Chisholm et al., 2006). In *P. syringae*, a plant pathogen, some of these TTSS mediated effectors are *avr* (avirulence) genes to which some hosts have developed resistance, often mediated by a single host gene that can recognize and neutralize the *avr* gene in a "gene for gene" manner (Flor, 1947). However, in a susceptible plant host these effectors can act on host protein substrates and interfere with host defenses and signaling by defense hormones such as salicylic acid (SA) (reviewed in Thomma et al. 2001). In the resistant host (cv. Xanthi) plants, we saw that several genes related to TTSS were down-regulated. This suggests that although a tobacco-PAO1 model has not been previously established, once PAO1 gains entry into susceptible tobacco plants, it can act as a true pathogen, utilizing TTSS and secreted virulence factors to compromise its host. Resistant *N. tabacum* cv. Xanthi, contains a dominant N-gene that can recognize an avirulence gene from tobacco mosaic virus (TMV), resulting in an SA-mediated hypersensitive response, restricted spread of the virus, and systemic resistance to TMV as well as other pathogens (Dinesh-Kumar, 1995). Although the susceptible cv. Samsun, was lacking a dominant N-gene, subsequent experiments demonstrating PAO1 resistance in both *N. tabacum* cv. Petite Havana SR-1::nn and a dominant N-gene containing transformant of this cultivar, pTG38, suggest that the N-gene does not play a role in resistance to PAO1. This was further confirmed by a lack of disease development in mutants that resulted in truncated forms of the N-gene protein. However, this does not rule out the possibility that there are other, unidentified resistance genes that convey resistance to specific effectors of PAO1. It does appear that the plant defense signaling compound, SA, plays a role in the differential resistance that is visible between the two cultivars, as we detected substantially more SA in leaf tissue of the

resistant cultivar in both infected and uninfected plants. SA may play a dual role in the defense of plants against *P. aeruginosa* and other human pathogens, as it is used by the plant to induce defense responses but also has direct negative effects on bacterial virulence (Prithiviraj et al. 2005a, b).

This study demonstrates the importance of nutrient availability in disease development. For instance, limitation of some nutrients, such as sulfate, appears to inhibit growth and subsequent pathogenicity. However, the limitation of others nutrients, like iron and phosphate, may stimulate the expression of virulence factors (Sokol et al., 1982; Liu, 1979). In summary, the results of this study suggest that the ability of plant defenses and innate immunity to modulate responses of the pathogen could be extrapolated to better understand pathogenesis in human hosts, and further emphasize the importance of the use of plant models to study human pathogens.

CHAPTER 3

Plant Root Exudates and Extracts Interfere with Bacterial Quorum Sensing

ABSTRACT

Inhibitors of bacterial quorum-sensing (QS) systems have the potential to be employed as novel therapeutic agents, as well as having promising agricultural and industrial applications. It has been shown that microbes and plants "communicate" with one another in the rhizosphere, with effects on both microbial populations and on the plant communities. Therefore, it is feasible to assume that plants may have picked up on the chemical language of bacteria and can mimic or interfere with this bacterial communication by generating their own chemical signals. This suggests that plant root exudates may be an excellent source of QS inhibitors. Here we have employed a screen and discovered that root exudates are indeed a rich source of potential QS inhibitors. Additionally, we have identified a novel class of QS inhibitors, triterpene saponins, and proposed a mechanism by which these compounds may be inhibiting QS.

INTRODUCTION

Although it was once thought that unicellular organisms like bacteria acted as individuals concerned only with sustenance and reproduction, it is now known that many of these organisms have developed sophisticated chemical languages that are still being decoded. In bacteria, this chemical communication has been termed "quorum-sensing" (QS), since a threshold level of bacteria is often required for the chemical signals, or autoinducers, to sufficiently accumulate (Fuqua et al., 1994). Once this "quorum" is reached, the bacteria are capable of acting coordinately to regulate expression of genes required for pathogenicity, transfer genetic material, initiate symbiotic relationships, and a variety of other functions (deKievet and Iglewski, 2000) This provides advantages to bacterial communities by permitting them to launch a coordinated attack on a host without prematurely notifying the host of the invasion, to adopt new protective growth forms like biofilms or spores, or to acquire favorable new genetic traits.

A variety of chemical classes have been utilized in this bacterial language. Many Gram-positive bacteria utilize short oligopeptide signals that induce a signal transduction cascade via a phosphorylation/dephosphorylation mechanism. The most common signals among Gram-negative bacteria are acyl homoserine lactones (AHLs) with varying lengths and modifications to the acyl side chains to confer species specificity. Other signal molecules that have been described include a 3-hydroxypalmitic acid methyl ester (Flavier et al., 1997), the *Pseudomonas* quinoline signal (PQS) (Pesci et al., 1999), butyrolactones (Gamard et al., 1997), and diketopiperazines (Holden et al., 1999). Although each bacterial species produces only one or a few of these AHLs there is some overlap between signaling molecules produced by different species and there are some

signals, like the AI-2-like signaling molecules produced by the *LuxS* gene family, which are thought to be used for interspecies communication (Surette et al., 1999).

QS systems play a key role in the expression of virulence determinants in many pathogenic bacteria. Furthermore, QS can be inhibited without any negative effects on the growth and metabolism of the bacteria relieving the selective pressures that result in resistance to so many antibacterial compounds. In addition, many bacteria exist in nature as antibiotic-resistant sessile communities or biofilms (Davies, 2003). However, biofilms of *P. aeruginosa* mutants deficient in one or both QS systems demonstrated decreased resistance to various antibiotics (Davies et al., 1998; Bjarnsholt et al., 2005). While the emerging complexity of many QS systems and the fact that no currently identified QS inhibitor (QSI) can block all quorum-controlled genes preclude QSI from becoming medical "silver bullets", it has been suggested that QS systems are a good potential target for combined antibacterial therapies (Rasmussen and Givskov, 2006).

Thus far, the majority of QS systems that have been characterized are in plant or animal associated bacteria (deKievet and Iglewski, 2000). Because of this long evolutionary association, and the fact that many potentially pathogenic bacteria are soil dwelling organisms, it stands to reason that plants, particularly root exudates, might serve as a rich source of QSI compounds. Indeed, it has been discovered that plants are capable of eavesdropping on bacteria by detecting and responding to their signaling molecules (Mathesius et al., 2003; Schuhegger et al., 2006). Since many leguminous plants form symbiotic relationships with bacteria, these are the best-documented cases of plant-microbe cross talk. In laboratory experiments, the model legume *Medicago truncatula* can differentially detect and respond to AHLs produced by a symbiont (*Sinorhizobium*

meliloti) and a pathogen (*P. aeruginosa*) (Mathesius et al., 2003). The protein expression patterns and root exudate composition of *M. truncatula* differed in response to the two AHLs tested, suggesting that the plants are able to differentiate between the bacteria based on their chemical signals. Other legumes, including *Pisum sativum* and *M. sativa* have also demonstrated the ability to secrete compounds that can act as signal mimics by either stimulating or repressing QS (Teplitski et al., 2000; Keshavan et al., 2005). QSIs have also been detected in extracts of carrot, chamomile, water lily, pepper, chili, and garlic (Rasmussen et al., 2005a) and several traditional medicinal plants (Adonizio et al, 2006, 2008), reinforcing the idea that plants may be a good source of these compounds.

The aim of this study was to screen the root exudates of several different plant families for QSIs using various reporter strains that respond to AHLs of differing acyl chain lengths. The rationale for using root exudates rather than using plant extracts is that root exudates have been shown to influence the composition of microbial communities under natural conditions (Broeckling et al., 2008). They also fluctuate in composition and quantity in response to different bacterial species and even different strains of a single species (Bais et al., 2004, 2005). Using various reporter strains, we were able to identify several plant species whose root exudates may contain QSI compounds. We chose one species, *Phytolacca americana*, to examine in greater depth and determined that there are several chemicals in the root exudates and leachates that may contribute to the QSI properties of this plant.

MATERIALS AND METHODS

Bacterial Strains

The *Pseudomonas aeruginosa* JY501 *lasB'-lacZ* indicator strain (Yarwood et al., 2005) was kindly provided by Dr. E. Peter Greenberg (University of Washington). Quorum sensing inhibitor indicator strain QSIS1 in an *E. coli* 1100 (*endA thi*) background (Rasmussen et al., 2005a) was provided by Dr. Michael Givskov (University of Denmark). The *Chromobacter violaceum* strain CV026 was obtained from Dr. Herbert Schweizer (Colorado State University). All cultures were maintained in 20% glycerol stocks at –80°C and cultured as described for each individual assay.

Plant Materials and Culture Conditions

Phytolacca americana seeds were kindly provided by Dr. Nilgum Tumer (Rutgers University, NJ) or propagated in greenhouses at Colorado State University. *Arabidopsis thaliana* Col-0 seeds were purchased from Lehle Seeds (Roundrock, Texas). Seeds of *Abelmoschus esculentus, Capsicum annuum, Solanum lycopersicum* and *Allium sativa* were purchased from Ferry Morse Seed Co. (Fulton, KY). Seeds of *Achillea millefolium, Eschscholzia californica,* and *Pimpinella anisum* were purchased from Plantation Products (Norton, MA). *Phaseolus vulgaris, Triticum aestivum, Rumex acetosa,* and *Lactuca sativa* seeds were obtained from Botanical Interests (Broomfield, CO). All other seeds were Burpee brand (Warminster, PA).

Root Exudate Preparations

Root exudates were collected from individual plants grown in 50 ml glass tubes with 3 ml of sterile dH_2O or in 500 ml flasks containing multiple plants and 200 ml sterile dH_20. Crude exudates were collected after one week and filtered with a 0.2 μm filter. Aliquots of the crude exudates were plated on LB agar and incubated at 30°C to ensure they were free of contamination. Exudates were extracted using 2 volumes of ethyl acetate. The organic phase was dried over anhydrous $MgSO_4$ and concentrated under compressed air. Extracts were resuspended in 1% DMSO. The aqueous phase was lyophilized and extracted as described by Keshavan et al (2005). Briefly, lyophilized exudates were resuspended in 50% MeOH (1ml $5mg^{-1}$) and the insoluble material was removed by centrifugation. An equal volume of 100% methanol and ¼ total volume of isopropanol were added and precipitates were filtered using Whatman #1 filter paper. The filtered extracts were concentrated and resuspended in sterile dH_2O at a concentration of 5 mg of dried residue ml^{-1}. All preparations were stored at –20°C prior to use.

QSIS1 Assay

Cells of QSIS1 were grown in ABT (AB medium supplemented with 2.5 mg of thiamine $liter^{-1}$) supplemented with 0.5% glucose and 0.5% casamino acids and incubated at 30°C. QSIS1 assays were conducted as described by Rasmussen et al. (2005a) with modifications. Briefly, ABT medium with 2% agar was melted and cooled to 45°C. A cocktail containing 100 μg ml^{-1} of ampicillin, 10 μM 3-*oxo*-C6-HSL (Sigma, St. Louis,

MO), 40 μg ml^{-1} X-gal, and 100 μM IPTG was added. Finally an overnight culture of QSIS1 grown in ABT minimal medium was added to a final culture OD_{600} = 0.002. Four wells of approximately 1 mm diameter were cut into solidified test plates and 50 μl $well^{-1}$ of test samples or control substances were added directly to each well. The system was calibrated using 100 μM 4-NPO and aqueous extracts of chamomile, compounds previously reported to exhibit QSI activity in this system (Rasmussen et al., 2005a). Additional controls for the various experiments included 1% DMSO, MS medium, sterile dH_20, and methanol.

CV026 Assay

Procedure was followed as described in McClean et al. (1997). Briefly, 100 ml of 1 % of melted LB agar was cooled to 45°C and seeded with 5 μM 3-*oxo*-C6-HSL (Sigma, St. Louis, MO) and 1 ml of an overnight culture of CV026. Seeded LB agar was overlaid on warmed LB plates and allowed to solidify. Root exudates were then pipetted into test wells as described for QSIS1. The presence of a white halo on a purple background was indicative of QSI or and a zone clear of bacteria was signified growth inhibition.

JY501 Assay

To examine the ability of root exudates to inhibit QS in a *P. aeruginosa* based reporter system that responds primarily to 3-*oxo*-C12-HSL, we used JY501, and AHL synthesis mutant carrying a chromosomal *lasB'-lacZ* reporter (Yarwood et al., 2005). JY501 cultures were added at an initial inoculum of OD_{600}=0.001 (Yarwood et al., 2005)

to FAB minimal medium amended with 2 μM C4-HSL (Sigma, St. Louis, MO) and 10 μM 3-*oxo*-C12-HSL (Cayman Chemicals, Ann Arbor, MI) that had been treated with 30% (v/v) of root exudates or sterile dH_2O and incubated at 37°C for amounts of time indicated. Reporter JY501 grown without added AHLs was used as a negative control. The amount of β-galactosidase (βGal) expressed was determined using a Miller assay following a modified version of the protocol of Zhang and Bremer (1995). Basically, after recording OD_{600} values, 20 μl of treated JY501 cells were removed and placed in 80 μl of a permeabilization solution (100 mM Na_2HPO_4, 20 mM KCl, 2mM $MgSO_4$, 0.8 mg ml^{-1} CTAB, 0.4 mg ml^{-1} sodium deoxycholate, 5.4 μl ml β-mercaptoethanol) to lyse bacterial cells. Six hundred microliters of a substrate solution (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 1 mg ml^{-1} o-nitrophenyl-β-D-galactoside (ONPG), 2.7 μl ml^{-1} β-mercaptoethanol) were added to each sample and incubated for 30 minutes at 30°C. Finally, 700 μL of 1 M Na_2CO_3 was added to each sample and vortexed to stop the reaction. Tubes were microfuged at high speed for five minutes to pellet cellular debris and 100 μl of supernatant from each sample was transferred to 96-well plates and read in a spectrophotometer at Abs_{420}. Miller units were then calculated using the following formula:

$$1000 \times (Abs_{420})/((Abs600) \times (\text{sample volume}) \times (\text{reaction time in minutes}))$$

Extraction of *Phytolacca americana* Root Material

Phytolacca americana roots were collected from Webster Springs, WV (lat: 38.47400, long:-80.41170, 1/7/2007). Roots were macrotomed and lyophilized prior to

being ground to a fine powder in a Waring blender with solidified carbon dioxide. The resulting powder was extracted three times in 100 ml MeOH per gram of dry weight and evaporated to dryness. The dried extracts were resuspended in H_2O and partitioned three times with ether. The aqueous layer was then extracted 3x with BuOH, resulting in three fractions (ether, aqueous, BuOH) that were tested for activity in the QSIS1 bioassay. All chemicals used were HPLC grade.

Flash and Thin Layer Chromatography

A normal phase silica column (RediSep, ISCO Lincoln, NE) was used on a CombiFlash Retrieve (ISCO, Lincoln, NE) system to separate out compounds present in the BuOH fraction. The lower phase of 90:10:10, 80:20:10 and 65:35:10 gradients of $CHCl_3$:MeOH:H_2O were used to collect 48 different fractions. Fractions were concentrated and spotted on normal phase TLC plates and developed using the lower phase of a 65:35:10 solution of $CHCl_3$:MeOH:H_2O and a vanillin spray. Similar fractions were combined into 10 fractions, dried, and resuspended in appropriate solvents for further analysis.

^{1}H- and ^{13}C-NMR

^{1}H and ^{13}C NMR spectra were performed on a Varian INOVA 400 instrument (Palo Alto, CA), operating at 400 MHz and 100 MHz respectively. The spectra were run in CD_3OD and compared with published spectra of related compounds.

RESULTS

Calibration of QSIS1 Reporter System

The QSIS1 system is an *E. coli* based reporter strain that contains a plasmid carrying the *luxR* gene and the *luxI* promoter fused with the *Serratia liquefaciens* gene *phlA*, which encodes a lethal protein resulting in growth arrest cell lysis in the absence of QS inhibitors (Rasmussen et al, 2005a). Quorum sensing inhibition is indicated by a ring of bacterial growth around the site of exogenously added QS inhibitory (QSI) compounds. Visualization of this growth is facilitated by providing the X-gal substrate and inducing the native *lac* operon with IPTG. Previous reports indicate that activation of QS, and thus bacterial inhibition, could be obtained by addition of 100 nM of 3-*oxo*-C6-HSL to 1 ml of overnight culture of QSIS1 (Rasmussen et al., 2005a). However, following this protocol, and using previously reported QS inhibitors 100 μM 4-NPO, toluene-extracted garlic, and methanol extracts of chamomile (Rasmussen et al., 2005a) as controls, we were unable to sufficiently activate QS to inhibit bacterial growth, resulting in plates that were covered in blue colonies. In order to activate QS in this system experiments were conducted where the medium containing antibiotics, X-gal, IPTG, and QSIS1 bacteria ($1x10^6$ cells ml^{-1}) were added without 3-*oxo*-C6-HSL. Several different concentrations of the 3-*oxo*-C6-HSL (100 nM-1 mM) were then added into 5 mm wells to determine the optimum concentration for the assays. Using this assay, it was determined that the minimum concentration of 3-*oxo*-C6-HSL required for QS activation was 5 μM (data not shown). After adjusting the protocol by adding 5 μM of 3-*oxo*-C6-HSL, QS inhibition was visible upon addition of garlic and chamomile extracts, and 100 μM 4-NPO to test wells (data not shown). Because water extracted chamomile

showed activity in this system, were easy to obtain, and inhibited QS without negative effects on bacterial growth, these extracts were chosen to serve as positive controls in further experiments.

Screening Plant Exudates for QS Inhibitors

Crude root exudates of 20 different plant species were screened for QS inhibition using the QSIS1 reporter strain. Exudates from seven of the 20 plants initially screened showed QS activity in the QSIS1 assay (Table 3.1). The seven active root exudates plus three of the inactive root exudates (carrot, lettuce and poppy) were then extracted with EtOAc and MeOH and retested in the QSIS1 reporter strain.

The very strong selection for mutations in the QSIS1 selector system mandates shorter incubation times (16-24 hours) of test plates to minimize false positive results (Rasmussen et al., 2005a). To ensure the validity of results, 100 μM 4-Nitro-pyridine-*N*-oxide (4-NPO) and polar chamomile extracts that were previously reported to have anti-QS activity (Rasmussen et al., 2005a), were used as positive controls and sterile distilled water and 1% DMSO were used as negative controls in each experimental repetition, and plates were read between 18-24 hours post-treatment. Using this qualitative assay, there was no visible QSI activity in any of the EtOAc extracts (Table 3.2). However, MeOH extracts that showed activity in the first screen as crude exudates retained this activity. Initially the root exudates were collected in MS medium; however, after extraction with MeOH, our MS medium negative controls showed activity in the QSIS1 reporter. All exudates were then grown in sterile dH_2O and retested, and all data shown is for these water-collected exudates. We found that the methanol extracts of *E. purpurea* and *C.*

annuum root exudates were somewhat active (showed activity in most repetitions but had smaller zones of growth), but root exudates of *A. thaliana, T. aestivum, P. americana, G. aristata,* and *P. vulgaris* consistently showed good inhibition of quorum-sensing in this system (Figure 3.1A). *Phaseolus vulgaris* was also growth inhibitory at higher concentrations (closer to the treatment area) and only had a QS inhibitory effect at lower concentrations. To eliminate the possibility that the ring of blue around test wells was an artifact of plant-produced β-galactosidases present in the root exudates rather than actual bacterial growth, all exudates were tested in plates that were not impregnated with the QSIS1 reporter strain. No hydrolysis of X-gal was noted. Furthermore, the QSI effect of the root exudates was determined to be a heat-stable metabolite rather than a protein as boiled exudates retained their QSI properties, although this treatment does not eliminate the possibility that short peptides present in the exudates may interfere with bacterial QS (Table 3.2).

To further characterize this activity, the MeOH extracts of the root exudates were tested in two additional reporter strains that respond to both short chain (CVO26) and long chain (JY501) acyl homoserine lactones (AHLs) (Table 3.2). The *Chromobacter violaceum* QS signal mutant, CV026, was used as a second qualitative screen for short-chain AHL inhibitors. Exogenously added C4- or C6-AHL's activates this strain's production of the purple pigment, violacein (Cha et al., 1998). A ring of white colonies around the area of treatment application indicates inhibition of QS (Figure 3.1B).

Exudates that did not show activity in the QSIS1 assay also had no activity in this system, and in general the results of this screen were very similar to those obtained using the QSIS1 reporter system. However, *T. aestivum* and *C. annuum* exudates were

exceptions, as they showed no QS inhibitory activity against this strain (Table 3.2). In addition, most of the active exudates were also growth inhibitory at the highest concentrations (closest to the test wells) and replicated experiments showed some inconsistency.

The final screening system used was a *Pseudomonas aeruginosa lasI-/rhlI*-strain containing a *lasB'-lacZ* reporter (JY501). The *lasB* gene is AHL-dependent and contributes to the production of elastase in *P. aeruginosa*. A qualitative assay expressing βGal activity in Miller units was used to determine the QSI activity of the various plant exudates. Controls consisted of JY501 amended with sterile dH_2O with (positive control) and without exogenously added AHLs (negative control). When bacteria were treated with 30% (v/v) of the extracted plant exudates, *P. vulgaris* was the only exudate tested that had an antibacterial effect against JY501, while none of the other exudates appeared to significantly negatively affect bacterial growth (Figure 3.3). In order to minimize its effects on growth *P. vulgaris* exudate was diluted by ¼, as determined by MIC (data not shown), prior to treatment of JY501. After treatment with 30% (v/v) of each exudate, βGal activity was measured at 12 and 24 hours. Using a Dunnet's 2-tailed test with 95% confidence, we found that βGal activity was significantly reduced relative to sterile dH_2O controls after addition of *P. americana, T. aestivum, E. californicum*, and *P. vulgaris* exudates (Table 3.2, Figure 3.2). However, the fact that high concentrations of *P. vulgaris* were growth inhibitory and that the βGal activity significantly increased between the 12-24 hour time points suggests that this inhibition may not be a direct effect on QS, or that it may be causing QS delays. In some individual experimental repetitions, *A. thaliana* and *G. aristata* also significantly reduced βGal production, however; these

results were not statistically significant when the means of individual experiments were pooled, and could be due to experimental error or possibly to a chemical instability of root exudate components over time.

Chemical Analysis of *P. americana* Root Exudates

Pokeweed root has been used in traditional medicine in both the United States and China so there is some preexisting knowledge of the chemistry of this plant. While no chemistry has been reported for the root exudates of *P. americana*, the roots, berries, and cell cultures contain several water-soluble triterpene saponins, which were initially thought to be a single compound referred to as phytolaccatoxin (Stout et al., 1964), but has since been resolved to reveal at least seven different structures (Suga et al., 1978; Takashi et al., 2001). Our initial attempts to separate compounds from pokeweed exudates using TLC were unsuccessful, so ^{1}H and ^{13}C-NMR spectra were obtained from the entire MeOH-extracted fraction (Appendix B). These data indicate that there is a mixture of several sugars in the exudates, and a number of specific peaks corresponded with glucose and xylose standard spectra obtained from the Biological Magnetic Resonance Data Bank (University of Wisconsin, Madison). There were also several peaks in both spectra that were suggestive of methyl groups belonging to a triterpene.

Bioassay Guided Fractionation of *P. americana* Root Extracts

Since preliminary NMR analysis of the root exudates suggested the presence of triterpene saponins and obtaining large amounts of exudates for chemical analysis is difficult, we used *P. americana* root extracts rich in saponins for further isolation and

analysis. Briefly, fresh roots were extracted following the protocol of Suga et al (1978), which yielded three fractions: aqueous, BuOH, and ether (Figure 3.3A and Figure 3.3B). All fractions were concentrated under compressed air and resuspended in appropriate solvents at a concentration of 1mg ml^{-1} and tested for activity in the QSIS1 reporter system. The ether layer fraction showed no activity, but both the aqueous and BuOH fractions had large rings of blue bacterial colonies, indicating QSI properties (Figure 3.4A). Neither fraction was growth inhibitory in this system at the tested concentration.

The saponin-rich BuOH fraction was chosen for further chemical separation and biological analysis. Using flash and thin layer chromatography, the BuOH layer was further separated into 10 fractions. Each fraction was tested for QSI activity in both the *luxR*-based QSIS1 and *lasB'-lacZ*-based JY501 reporter systems. None of the fractions showed any activity in the JY501 reporter (Table 3.3), but 3 of the more polar individual fractions and all ten fractions recombined were able to inhibit QS in the QSIS1 system (Table 3.3, Figure 3.4B), suggesting that there may be more than one active compound in these extracts.

^{1}H and ^{13}C NMR of BuOH Fractions

The BuOH fractions PAR3.3-3.4, PAR3.16-3.20, PAR3.21-3.22, and PAR4 were analyzed using ^{1}H NMR (Appendix B). The data for PAR 3.3-3.4 and 3.16-3.20 suggested that these fractions were nearly pure triterpene saponins that contained one and two sugar moieties, respectively. Since fraction PAR3.16-3.20 was active in the QSIS1 system, we also ran a ^{13}C NMR spectrum in order to determine an exact identity for this compound. Unfortunately, these spectral data did not exactly match any data previously

reported in the literature, precluding structural identification without further analysis. These triterpene saponins typically have five methyl groups present at positions C24, C25, C26, C27, C29, and occasionally a sixth methyl carbon is present at C31. The ^{13}C-methyl resonances of PAR3.16-3.20 in CD_3OD were most similar to those previously obtained for a 3-*O*-[β-D-glucopyranosyl-(1→2)-β-D-xylopyranosyl] phytolaccinic acid with a molecular formula of $C_{42}H_{66}O_{15}$ (Takahashi et al., 2001) or esculentoside L_1 (Strauss et al., 1995) (Table 3.4). A direct comparison of the C-methyl proton resonances of these two compounds with PAR3.16-3.20 is more difficult as the published spectrum were obtained in different solvent systems and at different resolutions. Although they were most similar to those published for 3-*O*-[β-D-glucopyranosyl-(1→2)-β-D-xylopyranosyl] phytolaccinic acid, the proton resonance suggest that these are not the same compound. The C-methyl proton resonances for PAR3.16-3.20 (400MHz, δ in CD_3OD) were δ: 0.788 (3H,s), 0.900 (3H,s), 1.128 (3H,s), 1.156 (3H,s), 1.252 (3H,s). The C-methyl proton resonances for 3-*O*-[β-D-glucopyranosyl-(1→2)-β-D-xylopyranosyl] phytolaccinic acid (600 MHz, δ in $CDCl_3$) were δ: 0.71 (3H,s), 0.82 (3H,s), 0.97 (3H,s), 1.12 (3H,s), 1.17 (3H,s) (Takahashi et al., 2001). Mass spectrum and other chemical analyses will likely be necessary to obtain an exact structure for PAR3.16-3.20. The PAR3.21-3.22 fraction was less pure than the other two, but still suggested the presence of one or more triterpene saponins. Finally, fraction PAR4, the most polar fraction appeared to be primarily sugars, mainly rhamnose. This was somewhat surprising as this fraction was also active and is probably most similar to the highly active aqueous layer that has not yet been chemically analyzed. When tested in the QSIS1

system, several pure sugars were not active and therefore, it is possible that there are other compounds present that are masked by the relative abundance of the sugars.

Table 3.1. Plant species whose root exudates were screened for QSI activity in the QSIS1 reporter strain. (-) = no inhibition, (+)= occasional or weak inhibition, (++) = strong inhibition.

Family	Species	Common Name	QSIS1 Activity
Alliaceae	*Allium cepa L.*	Onion	-
Apiaceae	*Daucus carota L.*	Carrot	-
Apiaceae	*Pimpinella anisum L.*	Anise	-
Asteraceae	*Achillea millefolium L.*	Yarrow	-
Asteraceae	*Echinacea purpurea*	Purple Coneflower	++
Asteraceae	*Gaillardia aristata*	Blanket flower	++
Asteraceae	*Lactuca sativa L.*	Lettuce	-
Brassicaceae	*Arabidopsis thaliana Heynh.*	Thale cress	+
Euphorbiaceae	*Euphorbia esula L.*	Leafy spurge	-
Fabaceae	*Medicago truncatula Gaertn.*	Barrel Medic	-
Fabaceae	*Phaseolus vulgaris L.*	Common bean	++
Fabaceae	*Pisum sativum L.*	Pea	-
Malvaceae	*Abelmoschus esculentus (L.) Moench*	Okra	-
Papaveraceae	*Eschscholzia californica Cham.*	California poppy	+
Phytolaccaceae	*Phytolacca americana*	Pokeweed	++
Poaceae	*Tritucum aestivum L.*	Wheatgrass	++
Polygonaceae	*Rumex acetosa L.*	Sorrel	-
Solanaceae	*Capsicum annuum L.*	Pepper	+
Solanaceae	*Solanum lycopersicum L.*	Tomato	-
Solanaceae	*Solanum melongena L.*	Eggplant	-

Table 3.2. Tested root exudates and their activity in various QS reporter strains. Symbols indicate: *growth inhibitory at higher concentrations, (-) = no inhibition, (+) = inhibition in some replicates, (++) = consistently inhibited QS in all experimental replicates.

Species	QSIS1	QSIS1			CV026	JY501
		MeOH Extracts	Boiled Extracts	No Bacteria		
Daucus carota	-	-	-	-	-	-
Gaillardia aristata	-	++	++	-	+*	-
Lactuca sativa	-	-	-	-	-	-
Echinacea purpurea	-	+	+	-	+*	++
Arabidopsis thaliana	-	++	++	-	-	-
Phaseolus vulgaris	-	++*	++*	-	+*	++*
Eschscholzia californica	-	-	-	-	-	++
Triticum aestivum	-	++	++	-	-	++
Capsicum annuum	-	+	+	-	-	-
Phytolacca americana	-	++	++	-	+*	++

Figure 3.1. (A) Using the broad based QSIS1 reporter (Rasmussen et al., 2005a) with chamomile extracts as a positive control and sterile dH_2O as a negative control, several plant root exudates showed QSI activity. (B) In a *C. violaceum* quorum-sensing assay, active exudates varied little from the QSIS1 reported but were generally growth inhibitory as well.

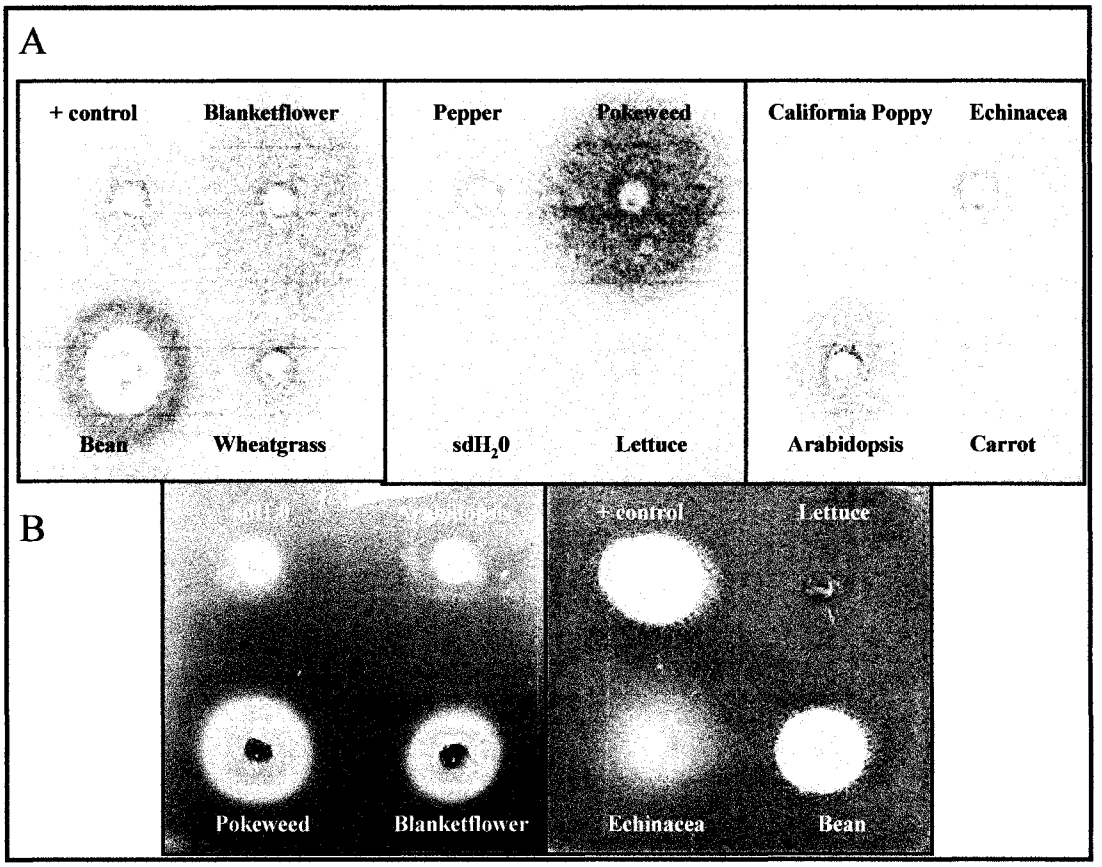


Figure 3.2. Relative measurement of β–Galactosidase (βGal) activity (expressed in Miller units) after a PAO1 *lasB'-lacZ* reporter (JY501) was treated with root exudates from various plant species. Several of the root exudates appeared to cause a delay in quorum sensing, as suggested by lower levels of βGal activity at 12 hours compared to 24 hours after treatment. However, exudates of both *P. americana* and *E. californica* showed lower levels of βGal activity at both time points, suggesting they contain quorum sensing inhibitors.

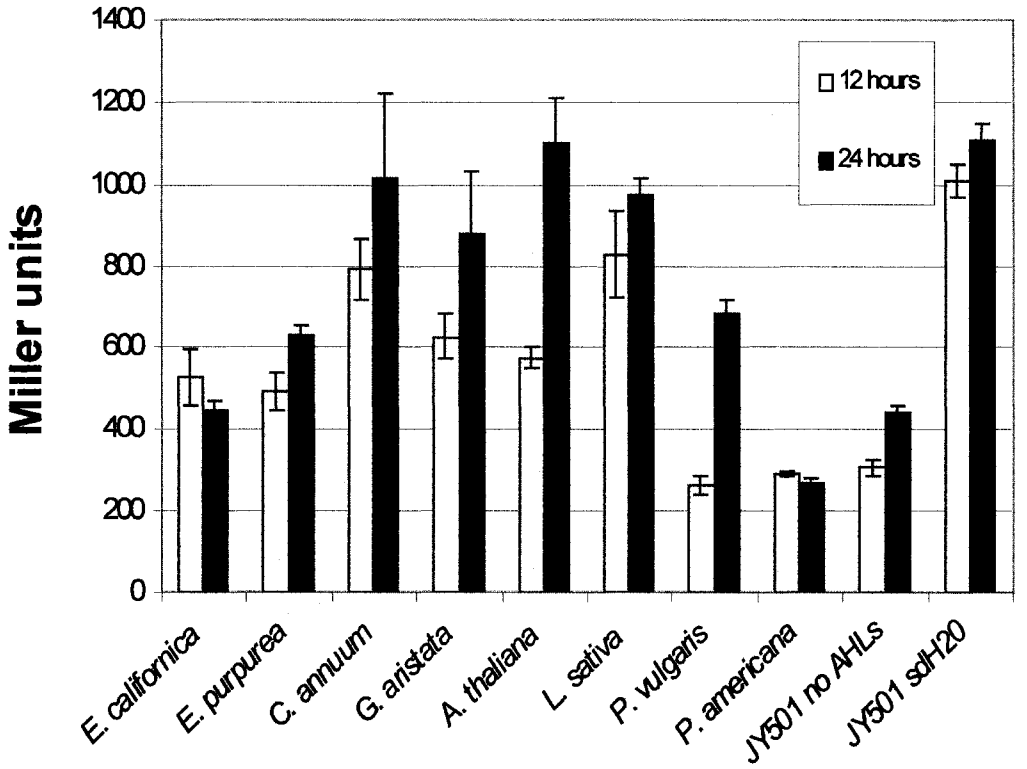


Figure 3.3. (A) Diagram of *P. americana* root tissue extraction protocol modified from Suga et al (1978). (B) Basic structure of the various triterpene saponins previously identified from *Phytolacca spp.*

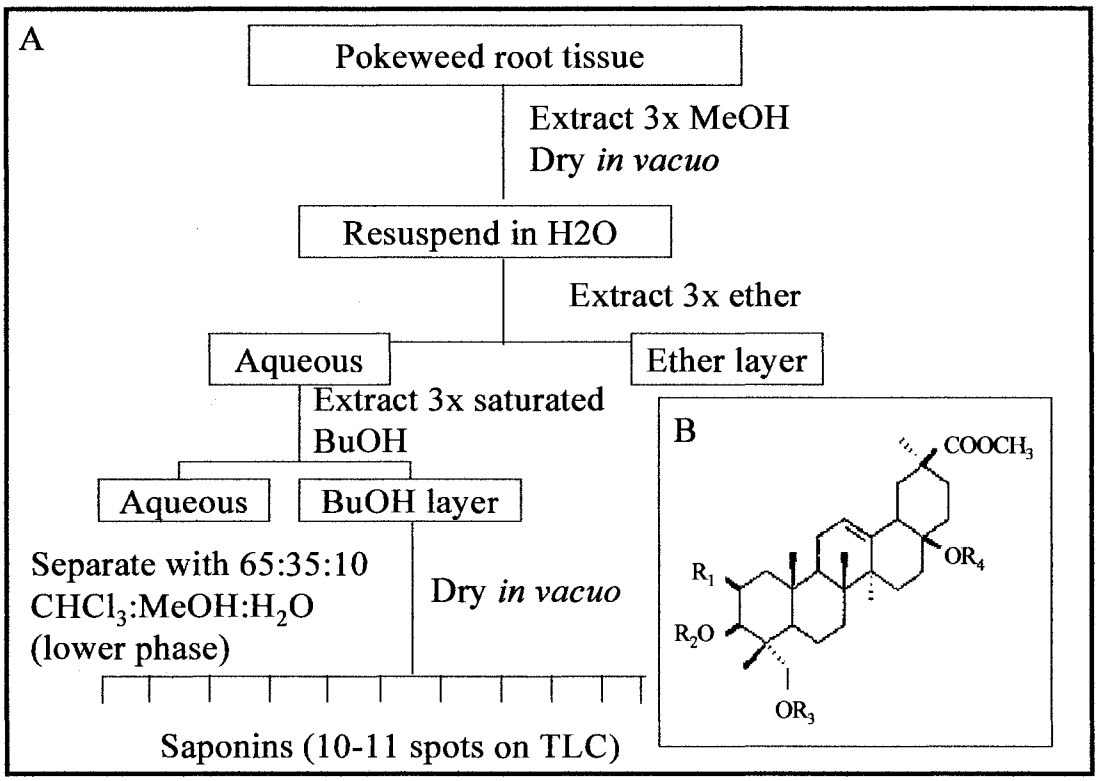


Table 3.3. Fractions obtained from BuOH extracts of *Phytolacca americana* roots. Activity in the QS reporter strains QSIS1 and JY501 is indicated by (+).

BuOH Fraction	QSIS1	JY501
PAR1.1-1.4	-	-
PAR2.1-2.8	-	-
PAR3.1-3.2	-	-
PAR3.3-3.4	-	-
PAR3.5-3.8	-	-
PAR3.9-3.15	-	-
PAR3.16-3.20	+	-
PAR3.21-3.22	+	-
PAR3.23-3.34	-	-
PAR4	+	-
recombined	+	-

Figure 3.4. (A) Using previously tested *P. americana* root exudates and the aqueous and BuOH extracts of *P. americana* roots showed QSI activity in the QSIS1 reporter. (B) Several individual partially purified saponin-containing fractions obtained by separation of the BuOH layer of *P. americana* had QSI activity.

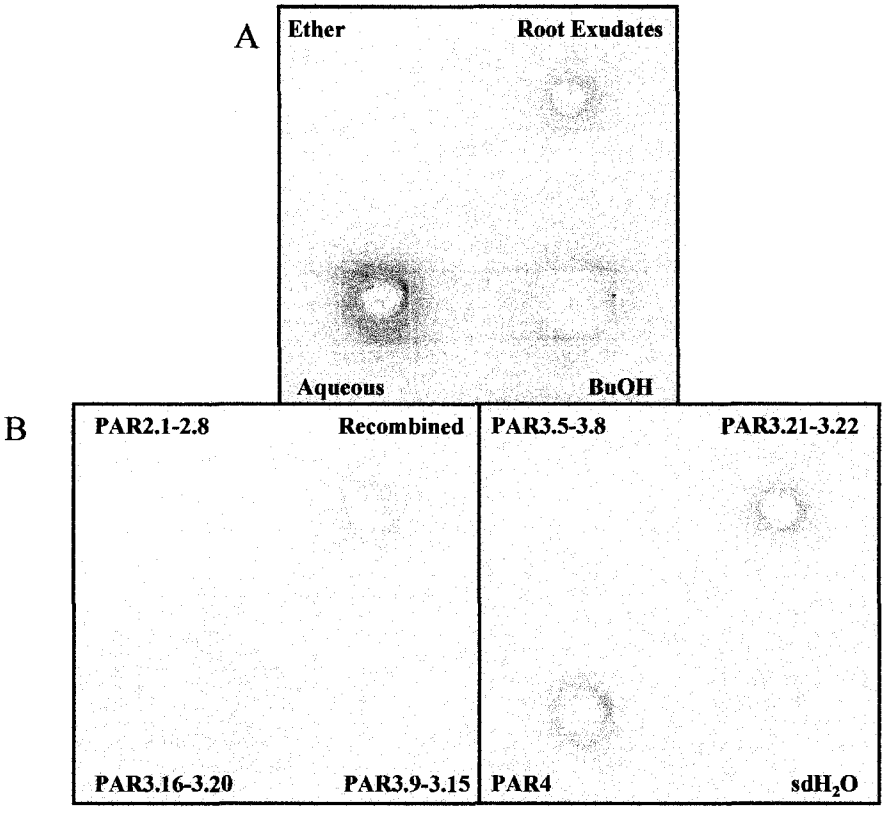


Table 3.4. Comparison of methyl C resonances of several triterpene saponins. ^{13}C-NMR data in CD_3OD for the PAR3.16-3.20 fraction from *P. americana* BuOH extracts compared with (1) 3-*O*-[β-D-glucopyranosyl-(1→2)-β-D-xylopyranosyl] phytolaccinic acid, (2) esculentoside B, (3) phytolaccoside E, (4) esculentoside S, (5) esculentoside L1, and (6) esculentoside (G) that were previously isolated from root cultures of *P. americana* (Takahashi et al., 2001).

	PAR3.16-3.20	*1*	*2*	*3*	*4*	*5*	*6*
C24	13.4	13.4	14.7	14.7	14.7	13.3	14.7
C25	16.3	16.4	17.5	17.5	17.6	16.5	17.6
C26	17.3	18.1	17.8	17.8	17.7	17.7	17.7
C27	26.3	26.4	26.4	26.4	26.3	26.3	26.3
C29	27.8	28.9	28.7	28.7	28.6	28.6	28.6

DISCUSSION

Bioprospecting of plant-produced metabolites in search of new therapeutic agents has been a successful area of research, resulting in a number of pharmaceuticals that are either derived from or structurally based on plant compounds. Digitoxin, taxol, ephedra, quinidine are all commercially successful examples of plant-derived pharmaceuticals, and there are countless plant metabolites, that reportedly demonstrate anti-microbial activity *in vitro* (Duke, 1993; Cowan, 1999). However, much of this bioprospecting has excluded examining the compounds present in root exudates, despite the fact that they have been shown to influence microbial populations in the rhizosphere (Broeckling et al., 2008) and to respond specifically to different bacterial species (Bais et al., 2005). This data, showing that 35% percent of the randomly chosen plant exudates that were screened had some level of QSI activity, validates the approach of examining root exudates for novel QSI compounds.

All of the active exudates in our screens were highly polar compounds as they preferentially partitioned to the aqueous phase during organic extraction, and none of the ethyl acetate extracts were found to be active. Many of these root exudates were found to be active in the QSIS1 system but were not active in the *P. aeruginosa* based *lasB'-lacZ* reporter strain. This is not surprising since the *lux* QS system used in the QSIS1 reporter strain responds to a wide spectrum of signaling molecules and therefore is likely to respond to a broader range of QSI compounds (Rasmussen et al., 2005a). By contrast, the *las* system in *P. aeruginosa* responds almost exclusively to 3-*oxo*-C12-HSL (Hentzer et al., 2002). The root exudates of several plant species, most notably *A. thaliana* and *P. vulgaris*, did show significantly increased expression of βGal at the 24-hour time point as

compared to the 12-hour time point, although untreated controls showed that βGal had been maximally induced by 12 hours. This suggests that some of the exudates that appear to be active in this system are merely resulting in delays in QS regulated expression. Yarwood et al. (2005) discovered that certain complex mediums contain an inhibitor or inhibitors that appear to be consumed by *P. aeruginosa*, resulting in delayed activation of many QS-controlled genes.

Phytolacca americana root exudates were chosen for further study because they showed activity in all of the reporter systems and because there is some existing knowledge of the chemistry of these plants. A number of triterpene saponins have been identified in the roots of *P. americana* (Johnson and Shimizu, 1974; Woo and Kang, 1976, Woo et al., 1978, 1980; Kang and Woo, 1980). Saponins are a heterogeneous group of compounds that are widely found among plants and have a number of reported activities including antimicrobial (Escalante et al., 2002), molluscicidal (Thilborg et al., 1994; Treyvaud et al., 2000), anti-cancer (Jeong et al., 2004), and antiinflamatory properties (Won et al., 2006). The amphipathic nature of saponins has resulted in their propensity to affect the solubility of other compounds and to act as emulsifying agents. It is this property that may be contributing to the QSI activity observed in this study.

Most of the QSIs that have been identified to date either inactivate the signaling molecules by chemical or enzymatic degradation (Yates et al., 2002; Dong et al., 2000, 2001) or are structurally similar to them, resulting in competitive binding to the cognate receptor molecules, targeting them for degradation or preventing them from forming stable DNA binding complexes (Rasmussen and Givskov, 2006). Triterpene saponins are not structurally similar to AHLs and would most likely be too large to bind the active site

of an AHL receptor. However, AHLs are thought to diffuse across plasma membranes and the emulsifying properties of saponins may increase the solubility of AHLs, preventing them from re-entering bacterial cells and binding to their target receptors. Since shorter chain AHLs would not cross the plasma membrane as easily as longer chain AHLs, the saponins present in the BuOH fractions may be sufficient to prevent re-entry of these molecules but may be less effective against the long chain AHLs which would more easily diffuse across the plasma membrane, explaining why the purified fractions showed no activity in the JY501 reporter system. Finally, the fact that there was biological activity in both the BuOH and aqueous fractions of *P. americana* suggests that there are potentially several classes of QSI compounds in the root exudates/extracts of *P. americana* that remain unidentified and that may have alternative modes of action. It is possible that compounds present in the aqueous fraction are responsible for the reduction of βGal activity in the JY501 reporter system.

CHAPTER 4

Phytolacca americana Root Exudates Result in Environmental Override of Quorum Sensing Regulated Genes.

ABSTRACT

Previous screening of root exudates from a range of plant species revealed that several of these plants secrete compounds that interfere with bacterial quorum-sensing (QS) systems. One of these plants, *Phytolacca americana*, or pokeweed, showed promise in multiple screening assays and was chosen for further study to determine its effects on the QS systems in *Pseudomonas aeruginosa*. Surprisingly, microarray analysis of global gene expression in *P. aeruginosa* revealed that the QS inhibitory effects of pokeweed root exudates noted in earlier assays were most likely not related to direct inhibition of QS systems. Instead, reduction in virulence factor production may be caused by environmental conditions in liquid culture resulting from the addition of the root exudates. Although the outcomes of microarray analyses are highly dependent on the time points for sampling and the number of replications, which were limited in this study by the high cost of these experiments, it does suggest that results of assays using QS reporter strains to identify inhibitors should be analyzed carefully and confirmed using additional tests.

INTRODUCTION

Pseudomonas aeruginosa is a ubiquitous Gram-negative bacterium that can infect a wide range of hosts, but is most commonly problematic in immunologically compromised patients, particularly those afflicted with cystic fibrosis (CF). In addition to a natural antibiotic resistance that *P. aeruginosa* has evolved from sharing a soil habitat with many antibiotic producing microbes, it often lives in sessile biofilms communities that are partially impervious to antibiotics, making these infections particularly challenging to treat. Depending on undetermined environmental cues, infection by *P. aeruginosa* can result in a minimally invasive chronic disease that is responsible for lung deterioration in CF patients or cytotoxic acute infections that often become systemic (Yahr and Greenberg, 2004). Formation of antibiotic resistant biofilms have been shown to be important in both types of infections (Costerton et al., 1999; Singh et al., 2000; Schaber et al., 2007), although a plethora of virulence determinants such as toxins and proteins that assist in host invasion are additionally employed in acute infections. In *P. aeruginosa*, the expression of many of these virulence factors is controlled by density dependant quorum-sensing (QS) systems (Latifi et al., 1995; Fuqua and Greenberg, 1998; Wagner et al., 2003; Schuster et al., 2003; Hentzer et al., 2003; Juhas et al., 2004).

Pseudomonas aeruginosa has an exquisitely complex, two-tiered QS system that regulates the timing and production of approximately 6% of the entire genome of the organism, including a plethora of virulence determinants (Whiteley et al., 1999). The *lasI* gene encodes a synthase for the 3-*oxo*-C12-homoserine lactone signal molecules, autoinducers that accumulate at sufficient cell densities and bind to the LasR cognate receptors (Passador et al., 1993; Pearson et al., 1994). These receptor-ligand pairs form

dimers that regulate transcription of hundreds of genes, including those in the *rhlI-rhlR* QS system (Wagner et al., 2003). The *rhlI-rhlR* system is responsible for the production of an additional signal molecule, C4-HSL, and its RhlR receptor. Together these QS circuits control transcription of an overlapping set of genes thought to be responsible for expression of virulence determinants and the ability of this pathogen to form mature biofilms (Davies et al., 1998; Parsek and Greenberg, 1999). In addition to this two-tiered complexity, there are a number of additional factors, such as the alternative sigma factor, RpoS, and the *Pseudomonas* quinoline signal (PQS) that are thought to be required for maximum induction of many QS-controlled genes (McKnight et al., 2000; Whiteley et al., 2000). There is also a homologue of the QS receptor molecules, QscR, which lacks a dedicated complementary AHL synthase, has been found to bind many AHLs with varying affinities, and which represses QS-controlled genes in its active state (Lequette et al., 2006). Furthermore, certain environmental conditions have been shown to override QS-control of some genes (Withers et al., 2001; Bollinger et al., 2001; Cornelis and Aendekerk, 2004), and even break down the hierarchy that is thought to exist between the two circuits (Duan and Surette, 2007).

Since the QS systems in *P. aeruginosa* control the production of so many virulence determinants and QS mutants have shown attenuated pathogenicity in a variety of infection models, they have become popular targets for drug discovery. Unfortunately, many of the QS inhibitory compounds identified to date are not nearly effective enough or are far too toxic to consider as potential therapeutics (Rasmussen and Givskov, 2006). An employed screen of plant root exudates to identify new QSI compounds revealed that these some of these exudates may be a good source of novel QSI's (Chapter 3). Most

notably, the exudates of *Phytolacca americana,* pokeweed, showed activity in several QS reporter strains. Pokeweed has been used medicinally in both eastern and western cultures, and has demonstrated antiviral and anticancer (Tumer et al., 1997; Uckun et al., 1997), molluscicidal (Thilborg et al., 1994; Treyvaud et al., 2000), and mitogenic properties (Glade et al., 1967). A ribosome inactivating protein called PAP (pokeweed anti-viral protein) is responsible for the antiviral properties of this plant (Irvin, 1983), and most of the other biological activities have been attributed to a range of triterpene saponins that have been identified in the roots and berries of the plant. Partially purified solutions of some of these triterpene saponins also showed anti-QS activity in a *lux*-based QS reporter strain, and crude exudates and aqueous root extracts from this plant had QS inhibitory activity in a *P. aeruginosa*-based *lasB'-lacZ* QS reporter (Chapter 3). Here we employ microbiological and molecular tests to evaluate the effectiveness and potential mode of action of pokeweed exudates on QS in *P. aeruginosa.*

MATERIALS AND METHODS

Plant Material and Bacterial Cultures

Seeds of *Phytolacca americana* were provided by Dr. Nilgum Tumer (Rutgers University) or collected from plants grown in greenhouses at Colorado State University. The *Pseudomonas aeruginosa* wild-type and JY501 *lasB'-lacZ* indicator strain (Yarwood et al., 2005) were donated by Dr. E. Peter Greenberg (University of Washington). The *P. aeruginosa lasI/rhlI* mutant strain PAO216 was created in our laboratory (Walker et al.,

2004). All cultures were maintained in 20% glycerol stocks at –80°C and cultured as described for each individual assay.

Collection and Extraction of Root Exudates

Phytolacca americana was grown in sterile dH_2O in 500 ml flasks containing multiple plants. Crude exudates were collected and filtered with a 0.2 μm filter prior to being plated on LB agar and incubated at 30°C to check for microbial contamination. Contaminant-free exudates were lyophilized and extracted as described by Keshavan et al (2005). Briefly, lyophilized exudates were resuspended in 50% MeOH (1ml $5mg^{-1}$) and the insoluble material was removed by centrifugation. An equal volume of 100% methanol and ¼ total volume of isopropanol were added and precipitates were removed by filtration. The filtered extracts were concentrated under compressed air and resuspended in sterile dH_2O at a concentration of 5 mg ml^{-1}. All preparations were stored at –20°C prior to use. Roots of *P. americana* were extracted according to the protocol published by Suga et al. (1978).

Dose-Response of PAO1 Using JY501 Reporter

To examine the ability of pokeweed compounds to inhibit QS in a *P. aeruginosa* based reporter system we used JY501, a QS signal mutant with a chromosomally integrated reporter (Yarwood et al., 2005). JY501 cultures were added at an initial inoculum of OD_{600}=0.001 to FAB minimal medium [1 mM $MgCl_2$, 0.1 mM $CaCl_2$, 0.01 mM Fe-EDTA, 0.15 mM $(NH_4)SO_4$, 0.33 mM Na_2HPO_4, 0.2 mM KH_2PO_4, 0.5 mM

NaCl, 10 mM sodium citrate] amended with 2 μM C4-HSL (Sigma, St. Louis, MO) and 10 μM 3-oxo-C12-HSL (Cayman Chemicals, Ann Arbor, MI) and treated with 0.5-30% (v/v) of root exudates or aqueous root extracts and incubated at 37°C for 24 hours. Negative controls consisted of JY501 without added AHLs and both negative and positive controls were volume adjusted with sterile dH_20. The amount of βGal expressed was determined using a Miller assay following a modified version of the protocol of Zhang and Bremer (1995). Briefly, after recording OD_{600} values, 20 μl of treated JY501 cells were removed and placed in 80 μl of a permeabilization solution (100 mM Na_2HPO_4, 20 mM KCl, 2mM $MgSO_4$, 0.8 mg ml^{-1} CTAB, 0.4 mg ml^{-1} sodium deoxycholate, 5.4 μl ml β-mercaptoethanol) to lyse bacterial cells. Six hundred microliters of a substrate solution (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 1 mg ml^{-1} o-nitrophenyl-β-D-galactoside (ONPG), 2.7 μl ml^{-1} β-mercaptoethanol) were added to each sample and incubated for 30 minutes at 30°C. Finally, 700 μL of 1 M Na_2CO_3 was added to each sample and vortexed to stop the reaction. Tubes were microfuged at high speed for five minutes to pellet cellular debris and 100 μl of supernatant from each sample was transferred to 96-well plates and read in a spectrophotometer at Abs_{420}. Miller units were then calculated using the following formula:

$$1000 \times (Abs_{420})/((Abs600) \times (\text{sample volume}) \times (\text{reaction time in minutes}))$$

Elastase and Protease Assays

Qualitative plate assays for total protease and elastase activity were performed as described by Brint and Ohman (1995). To determine protease activity, cultures of PAO1

were stab inoculated on medium containing 0.8% nutrient broth and 1.5% powdered skim milk amended with 15% (v/v) *P. americana* root exudates or sterile dH_2O and were incubated at 37°C for 12 to 24 h before visual inspection for a zone of clearing. The same procedure was followed for elastase activity except that 0.5% elastin (Sigma, St. Louis, MO) was incorporated into the medium in place of 1.5% skim milk and cultures were incubated for 48 h. Protease activity was quantified using a modification of the method described by Greene et al. (1989). Bacterial cultures were grown with and without added root exudates in 5 ml of 5% peptone and 0.25% tryptic soy broth (PTSB) at 37°C for 24 h. The supernatants were collected and filter purified using a 0.22-μm nylon filter. A 100-μl aliquot of supernatant was then added to reaction mixtures containing 0.8% azocasein (Sigma, St. Louis, MO) in 500 μl of 50 mM K_2HPO_4, pH 7. Reaction mixtures were incubated at 25°C for 3 hours. The reaction was stopped by adding 0.5 ml of 1.5 M HCl, and the mixture was placed on ice for 30 min and then centrifuged. After addition of 0.5 ml of 1 N NaOH, the OD_{440} was recorded. To determine elastase activity, 100 μl of supernatant from the 24-hour PTSB cultures was added to tubes containing 1 ml of 10 mM Na_2HPO_4, pH 7, and 20 mg of elastin-Congo red (Sigma, St. Louis, MO). Reaction mixtures were incubated with agitation for 4 hours at 37°C. Tubes were then centrifuged, and the OD_{495} was determined.

Microarray Analysis

Bacterial cultures were grown in FAB minimal medium [1 mM $MgCl_2$, 0.1 mM $CaCl_2$, 0.01 mM Fe-EDTA, 0.15 mM $(NH_4)SO_4$, 0.33 mM Na_2HPO_4, 0.2 mM KH_2PO_4, 0.5 mM NaCl, 10 mM sodium citrate] containing 15% (v/v) *P. americana* (v/v) root

exudates (treatment) or 15% (v/v) sterile dH2O (controls) and grown to OD_{600}=1.5. Bacterial cells were collected by centrifugation, and pellets were flash frozen in liquid nitrogen prior to RNA isolation using a Ribopure RNA kit (Ambion Inc., Austin, TX) according to the manufacturer's instructions with slight modifications. Bacterial cells were vortexed 3x for 10 minutes to increase efficiency of mechanical cell lysis, resulting in higher concentrations of recovered RNA. The additional DNAse I treatment was included to minimize contamination by chromosomal DNA. Samples were concentrated using the RNeasy MiniElute Cleanup kit following the manufacturer's instructions (Qiagen Inc., Valencia, CA). The concentration of RNA was assessed using a Nanodrop system (Nanodrop, Wilmington, DE) and RNA integrity was determined by agarose gel electrophoresis and by 28S/18S ratio on an Agilent 2100 bioanalyzer (Agilent, Palo Alto, CA). Preparation of cDNA, labeling, and hybridization to Affymetrix *P. aeruginosa* GeneChips (Affymetrix Inc., USA) were conducted at Asuragen Inc., (Austin, TX) using the following instruments: GeneChip 640 hybridization oven, GeneChip 450 Fluidics Station, and a high-resolution GeneChip 3000 scanner (GeneChip, Santa Clara, CA). Microarray hybridizations were performed in triplicate using RNA/cDNA obtained from concurrently conducted independent experiments. The data analyses were performed with GeneSpring 7.2 (Silicon Genetics, Redwood City, CA) DNAChip Analyzer. The data were normalized per chip by dChip invariant set normalization, and each gene was normalized to the median measurement taken for that gene across all samples. Microarray analyses were performed in duplicate from independent experiments. Genes exhibiting a three-fold or greater change compared to controls and a P value of ≤ 0.05 were considered

significantly differentially expressed. All gene annotations are from the Pseudomonas Genome Project website.

RESULTS

Dose Response Curves of Pokeweed Exudates/Extracts

Root exudates of *P. americana*, or pokeweed, showed anti-QS activity in previous screens using various reporter systems and was chosen for further study. Dose-response curves were calculated using the JY501 *lasB'-lacZ* reporter strain. Methanol extracted pokeweed exudates and aqueous root extracts (5 mg of dry extract per 1ml of water) were tested at concentrations ranging from 0.5-30% (v/v) for inhibition of βGal expression. Root exudates of *P. americana* had no growth inhibitory effects on JY501 at any concentration and in fact, stimulated bacterial growth at most concentrations (Figure 4.1), probably due to the additional carbon sources supplied in the form of secreted sugars. Conversely, aqueous root extracts of *P. americana* were growth inhibitory at 20%-30% (v/v) concentrations, but otherwise showed similar concentration-dependent inhibition of *lasB'-lacZ* expression as the root exudates. However, because the growth inhibition that was noted at higher concentrations might indicate effects on the bacteria not related to QS, only root exudates were used for subsequent experiments.

Induction of *lasB'-lacZ* in *P. aeruginosa* JY501 was statistically significantly inhibited relative to untreated JY501 by the aqueous extracts of *P. americana* root exudates at concentrations ranging from 5%-30% (v/v) as determined by using Dunnett's 2-tailed comparison (Figure 4.1). At 30% (v/v) *P. americana* exudates reduced the induction of *lasB'-lacZ* by more than 50%. It should be noted that since plant roots may

vary in their production and secretion of active compounds, activity was tested on each new batch of exudates and extracts and the data shown represents a typical dose-response curve.

Pokeweed Exudates Decrease Elastase but Not Protease Production

Pokeweed exudates tested in the JY501 reporter strain had a negative impact on transcription of the *lasB* gene, which results in the expression of elastase. However, because this reporter is an AHL signal mutant that requires exogenously added AHLs for QS activation, it was necessary to examine the effect of pokeweed exudates on elastase and protease production in wild-type PAO1. Hydrolysis of elastin and casein was measured in pokeweed exudate-treated and untreated PAO1 and in PAO216, a *lasI-/rhlI-* double mutant using both qualitative plate assays and quantitative colorimetric assays. As expected, the plate assay showed reduced elastase activity in pokeweed-treated PAO1 relative to untreated controls, and this was confirmed by the quantitative assay which showed an ~30% decrease in elastase activity (Figure 4.2A). These results were consistent with those obtained for 15% (v/v) of pokeweed exudates in the dose-response curve using the JY501 reporter. However, if QS were being inhibited, reduction in additional virulence factors such as protease would also be expected. Surprisingly, protease activity measured by casein hydrolysis was not reduced by the addition of the pokeweed exudates in either the qualitative or quantitative assays (Figure 4.2B).

Microarray Analysis of PAO1 Treated with Pokeweed Exudates

Global gene expression in PAO1 in the presence and absence of pokeweed root exudates was examined to more comprehensively explore how these exudates were

affecting *P. aeruginosa*. Treated and untreated cultures of PAO1 were extracted for RNA, and absolute expression values of treated relative to untreated samples were compared. A p-value of ≤ 0.05 and a fold change ≥ 3 was required for inclusion in the analysis. Only 16 of the more than 200 genes that were previously shown to be QS-controlled (Whiteley et al. 2001, Hentzer et al. 2003), or <10% of the total QS regulon, were down-regulated in bacterial cells grown with pokeweed exudates (Table 4.1). Furthermore, all of these genes were down-regulated at a magnitude of 3-10 fold, while previously reported QS inhibitors have been able to down-regulate as much as 34%-37% of the QS regulon and some genes were repressed by as much as 75-150 fold (Rasmussen et al., 2005a). In addition, there were 7 QS controlled genes that were significantly up-regulated in the pokeweed exudate treated plants, including 3 genes that are thought to be involved in AHL synthesis (Table 4.1). These data do not provide strong support for the hypothesis that pokeweed exudates are able to directly interfere with QS in *P. aeruginosa,* although because of the low replication number of this experiment and the chemical variability in individual batches of root exudates from pokeweed, the possibility of QS-inhibition should not be entirely excluded.

Further examination of the microarray data suggests that the root exudates of pokeweed may be interfering with the metabolism of PAO1 in such a way that at least some QS–controlled virulence factors may be attenuated. There were at least 19 genes involved in nitrogen metabolism that were up-regulated in the exudate-treated cultures (Table 4.2). Among these, 11 genes were previously reported to be up-regulated in cultures grown aerobically with added nitrate, as compared to cultures grown aerobically

without nitrate; and only one gene, *aroE*, was up-regulated in cultures grown anaerobically relative to those grown aerobically with nitrate (Filiatrault et al., 2005).

Also of note, our data revealed that at least 17 genes that are involved in iron acquisition, specifically in the synthesis of two siderophores, pyoverdine and pyochelin, were also down-regulated in PAO1 grown with pokeweed exudates (Table 4.3). Pyoverdine is a greenish pigment that enables acquisition of Fe (III) from the environment (Meyer and Stintzi, 1998) and is important for virulence of *P. aeruginosa* (Meyer et al., 1996). *PvdS*, an alternative sigma factor is necessary for expression of several *pvd* genes that are required for pyoverdine synthesis was down-regulated by nearly 10–fold in the presence of pokeweed exudates. The siderophore pyochelin is derived from salicylate and two cysteines, and also thought to contribute to the virulence of *P. aeruginosa* (Cox, 1982). Two of the genes that we saw down-regulated were *pchEF*, which are responsible for the condensation of salicylate with the cysteine residues (Reimmann et al., 1998), although enzymes required earlier in this pathway, such as those encoded by the *pchDCBA* operon, were unaffected.

Figure 4.1. Pokeweed root exudates and extracts were tested at concentrations ranging from 0.5-30% (v/v) of crude extract for inhibition of βGal expression in a *lasB'-lacZ* reporter strain of PAO1 (JY501). βGal activity is expressed in Miller units and represented by vertical bars. Horizontal lines connect points representing the average OD_{600} for each treatment.

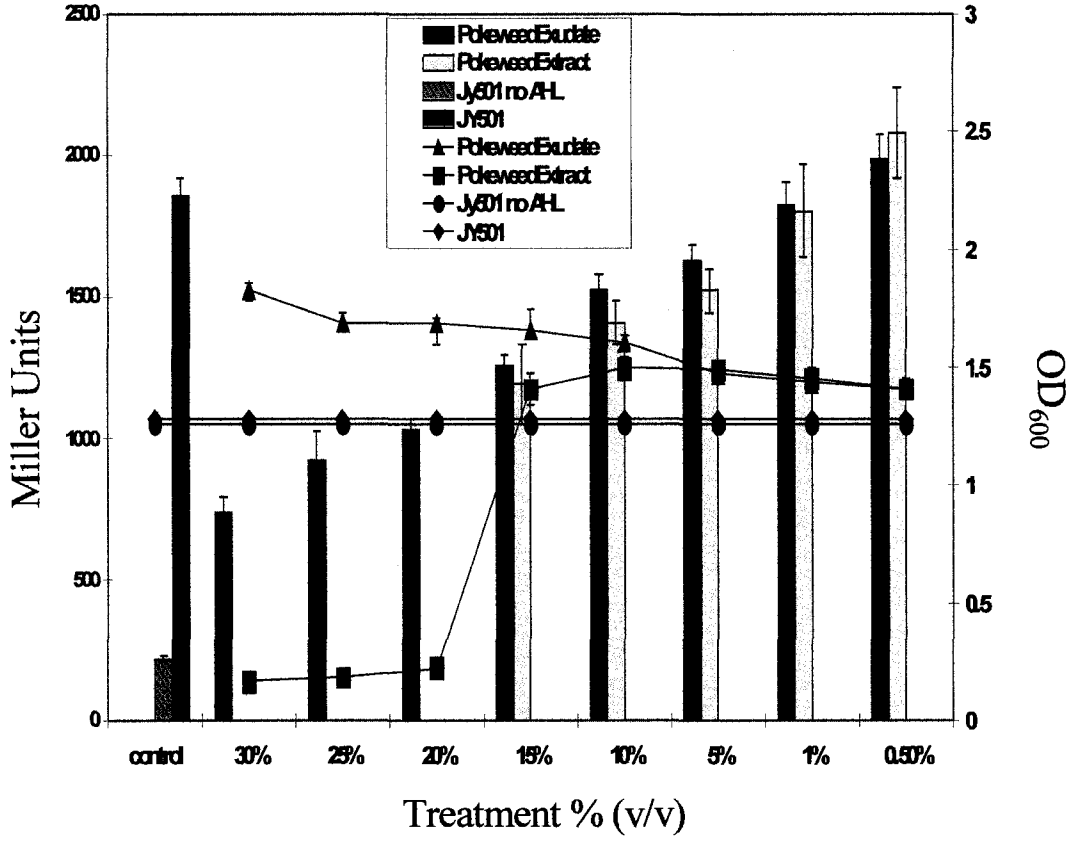


Figure 4.2. (A) Qualitative and quantitative assays show a reduction in elastase activity in pokeweed root exudate treated PAO1. Photos in the first column show wild-type PAO1 and the second column are PAO216 a QS double mutant deficient in the ability to produce AHLs. (B) Assays for protease activity show that this activity was unaffected by pokeweed treatment.

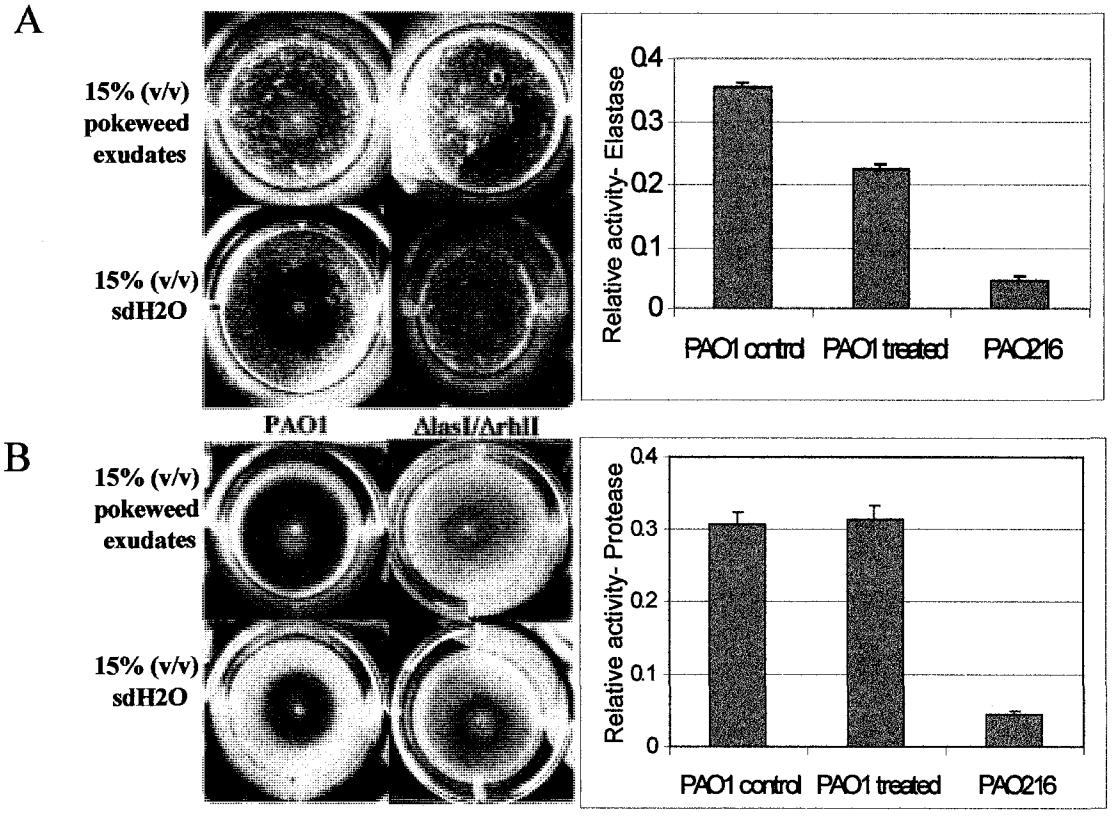


Table 4.1. QS controlled genes that were differentially regulated in PAO1 after exposure to *P. americana* root exudates. The QS regulon has previously been identified in microarray studies (Whiteley et al. 2001, Hentzer et al. 2003).

Gene Name	Fold Change	p-value	Predicted function
PA0122	-4.2	0.022	conserved hypothetical protein
PA0144	-5.0	0.021	nucleotide transport and metabolism
lasI	-4.7	0.005	autoinducer synthesis protein
PA1869	-6.8	0.037	probable acyl carrier protein
PA2030	-6.8	0.028	hypothetical protein
PA2031	-8.0	0.023	hypothetical protein
PA2331	-4.2	0.006	hypothetical protein
PA3181	-6.5	0.024	2-keto-3-deoxy-6-phosphogluconate aldolase
PA3182	-5.5	0.019	6-phosphogluconolactonase
zwf	-9.3	0.004	glucose-6-phosphate 1-dehydrogenase
PA3190	-8.1	0.021	probable binding component of ABC sugar transporter
edd	-4.5	0.030	phosphogluconate dehydratase
gapA	-8.0	0.025	glyceraldehyde 3-phosphate dehydrogenase
PA3361	-6.8	0.024	fucose-binding lectin PA-IIL
rhlR	-3.0	0.030	transcriptional regulator
PA4778	-6.9	0.045	probable transcriptional regulator
PA1659	6.0	0.04	hypothetical protein
PA1665	7.2	0.003	hypothetical protein
PA1666	5.3	< 0.001	hypothetical protein
PA1667	7.2	0.032	hypothetical protein
PA3332	4.6	0.043	conserved hypothetical protein
fabH2	3.6	0.004	3-oxoacyl-[acyl-carrier-protein] synthase III
PA3334	4.9	0.042	probable acyl carrier protein

Table 4.2. Genes involved in denitrification that were up-regulated after exposure to P. *americana* root exudates. Genes in bold face type were similarly regulated under aerobic conditions but with additional nitrate added to the culture medium, and * indicates that it was similarly regulated under anaerobic conditions (Filiatrault et al., 2005).

Gene Name	Fold Change	p-value	Predicted function
*aroE**	3.070	< 0.001	shikimate dehydrogenase
nirF	14.100	0.037	heme d1 biosynthesis protein NirF
nirC	14.200	0.008	probable c-type cytochrome precursor
nirM	11.400	0.050	cytochrome c-551 precursor
nirS	10.200	0.015	nitrite reductase precursor
norC	9.900	0.050	nitric-oxide reductase subunit C
norB	12.300	0.014	nitric-oxide reductase subunit B
napB	3.900	0.038	cytochrome c-type protein NapB precursor
napA	4.100	0.027	periplasmic nitrate reductase protein NapA
nosR	10.300	0.021	regulatory protein NosR
nosZ	10.000	0.050	nitrous-oxide reductase precursor
narJ	7.600	0.002	respiratory nitrate reductase delta chain
narH	14.200	< 0.001	respiratory nitrate reductase beta chain
narG	17.600	0.014	respiratory nitrate reductase alpha chain
PA0459	4.100	0.012	probable ClpA/B protease ATP binding subunit
PA2691	4.300	0.013	conserved hypothetical protein
PA3880	6.100	0.009	conserved hypothetical protein
PA3913	7.900	0.025	probable protease
PA4610	5.500	0.035	hypothetical protein

Table 4.3. Genes related to iron acquisition that were down-regulated in PAO1 after exposure to *P. americana* root exudates. Many of these genes are involved in the biosynthesis of pyoverdine and pyochelin, two classes of siderophores produced for *P. aeruginosa* for iron acquisition.

Gene Name	Fold Change	p-value	Predicted Function
pvdA	-10.3	0.044	L-ornithine N5-oxygenase
pvdP	-3.7	0.036	pyoveridine synthesis protein PvdP
pvdF	-3.6	0.049	pyoverdine synthetase F
pvdL	-6.9	0.036	pyoveridine synthesis protein PvdL
pvdG	-7.1	0.002	pyoveridine synthesis protein PvdG
pvdS	-9.3	0.037	transcription regulator, sigma factor
hasAp	-4.3	0.014	heme acquisition protein HasAp
PA4220	-11.0	0.036	hypothetical protein
fptA	-5.8	0.015	Fe(III)-pyochelin outer membrane receptor precursor
PA4222	-9.8	0.038	probable ATP-binding component of ABC transporter
PA4223	-10.9	0.047	probable ATP-binding component of ABC transporter
pchG	-8.8	0.048	pyochelin biosynthetic protein PchG
pchF	-12.7	0.039	pyochelin synthetase
pchE	-8.5	0.041	dihydroaeruginoic acid synthetase
PA4358	-5.6	0.033	probable ferrous iron transport protein
hitA	-5.4	0.015	ferric iron-binding periplasmic protein HitA
PA5217	-4.3	0.017	probable binding protein component of ABC iron transporter

DISCUSSION

Although the exudates of *P. americana* showed promising QSI activity in initial screens using reporter strains, the dose-response curve indicated that even if found to be non-toxic to humans, these exudates would not result in a viable QS-targeted therapeutic product. Although relatively crude, high concentrations (30% v/v) of exudates were required for only a 50% reduction in *lasB'-lacZ* induction. This is quite high when compared to the QSI seen with other crude extracts, such as garlic exudates that required only 2% (v/v) for a 50% reduction of *lasB* induction (Rasmussen et al., 2005a). Even at this level of inhibition, it was calculated that one would need to consume ~50 garlic bulbs per day to enjoy any QSI benefits from garlic extracts (Rasmussen and Givskov, 2006).

Additional analysis suggested that the exudates of pokeweed might not even have a direct effect on QS in *P. aeruginosa.* Wild-type PAO1 revealed that no reduction in QS-controlled protease production resulted from exposure to pokeweed root exudates. The *lasI-lasR* QS circuit largely controls production of both elastase and protease, and decrease in one enzyme but not the other indicates that pokeweed exudates may have specific effects on some QS-controlled genes but not direct effects on the main QS regulatory network. It has been shown that *rhlI-rhlR* and *Pseudomonas* quinoline signal (PQS) are also required for full induction of *lasB* (McKnight et al., 2000) and it is possible that the observed reduction in elastase production was due to interference with one of these additional regulatory layers.

Evidence increasingly indicates that QS-mediated cell-to-cell signaling in bacteria is influenced by environmental factors other than cell density. In *P. aeruginosa,* modulation of the levels of many virulence factors, including *lasB,* is thought to be due to

environmental overrides of the QS regulatory network. For example, cyanide production is increased under conditions of low oxygen (Pessi and Haas, 2000), and Filiatrault et al., (2005) reported that expression of a number of virulence factors was influenced by the addition of nitrate to culture medium. Another environmental factor that reportedly can override QS signaling is iron. It has been shown that limited iron and abundant O_2 favor the maximum induction of many QS controlled genes, including *lasB*, and some QS controlled genes can be expressed in QS signal mutants by manipulating nutrient content of the growth medium (Iglewski et al., 1990; Sokol et al., 1982). Conversely, high amounts of iron can lead to relative repression of QS-regulated genes such as *lasB*, even in the presence of adequate QS signal (Bollinger et al., 2001). The current microarray data suggest that the addition of the pokeweed exudates produces culture conditions that are rich in both nitrate and iron and possibly limited in oxygen; conditions that favor the repression of various QS-controlled genes. Interestingly, there was no pokeweed exudate-induced reduction in *lasB* expression noted in our microarray data. There are several possible explanations for this; among them are the obvious lack of adequate replication in the microarray experiments, and differences in the experimental growth conditions (stationary 96-well plates versus shaken cultures). However, there was a decrease in *lasI* expression, which could be related to an abundance of available iron in the culture medium as *lasI* was shown to increase under conditions of iron-deprivation (Bollinger et al., 2001).

Based on the available microarray data, it can be conclude that some QS-controlled genes in *P. aeruginosa* are being affected by the root exudates of pokeweed, although likely not through direct interference with the *las* or *rhl* QS circuits, but rather

through environmental influences. This does not exclude the possibility that the QS-inhibition of pokeweed exudates observed in *luxR*-based reporter systems is due to direct interference of the QS regulatory network. In fact, a cyclic disulphur compound identified in garlic extracts was strongly antagonistic in *luxR*-based QS reporters but showed no activity against *P. aeruginosa* QS (Rasmussen et al., 2005a; Persson et al., 2005). Similarly, the natural halogenated furanones that were isolated from the alga *Delisea pulchra* also had no effect although synthetic derivates based on the natural structure were quite effective in inhibiting both of the *P. aeruginosa* QS systems (Manefield et al., 2002; Hentzer et al., 2002, 2003; Hjelmgaard et al., 2003). In conclusion, these microarray data should be viewed cautiously as these experiments were only performed in duplicate and at a single time-point. The outcome of microarray analysis is strongly dependent on the number and timing of samples taken throughout the cell cycle. It has been determined that QS-controlled genes are expressed in a continuum throughout the growth cycle rather than at some threshold population as previously believed (Schuster et al., 2004; Hentzer et al., 2003). Therefore, it is important to examine samples at varying time-points to get a better picture of the effects a potential QSI may have on QS regulation.

CHAPTER 5

Plant Root Exudates Influence Competitive Outcomes Between Rhizosphere Bacteria, *Pseudomonas aeruginosa* and *Agrobacterium tumefaciens*

ABSTRACT

In the rhizosophere environment microbes exist as multispecies communities in association with plant roots. The balance of these relationships can have numerous consequences to the plant, affecting soil properties, the plant's ability to obtain and utilize nutrients, and determining whether some of these soil microbes will become pathogenic to the plant. The relationships between the soil microbes are also affected by the presence of the plant. However, competition between multiple microbial species is rarely examined in this context. Here we begin to explore how the competitive relationship between two soil dwelling microbial populations, *Pseudomonas aeruginosa* and *Agrobacterium tumefaciens*, is effected by the presence of *Arabidopsis thaliana* and its array of root-exuded secondary metabolites. We also begin to examine how quorum sensing, which has been determined to be an important factor in both microbial competition and pathogenic relationships with plants, is involved in this complex system.

INTRODUCTION

The revelation that bacteria can act as coordinated communities has changed the way microbiology is studied. To gain a better understanding of microbial behavior it is becoming increasingly necessary to determine how different components of natural environments influence the ability of a population to obtain nutrients and reproduce. Multiple microbial species coexist in the natural environment and compete for existing resources. A competitive edge can therefore be gained through mechanisms such as a faster growth rate (Li and Alexander, 1986; An et al., 2006), ability to utilize diverse carbon sources (Knee et al., 2001), the use of secreted enzymes to degrade signaling molecules of competing species, and production of antibiotics (Bais et al., 2004). New research is increasingly revealing that bacteria behave very differently in different environments. For instance, bacterial quorum-sensing (QS) in *Pseudomonas aeruginosa* has been studied extensively and careful research has revealed a hierarchical regulatory mechanism between two overlapping QS circuits (reviewed in Smith and Iglewski, 2003). However, a recent report has challenged this regulatory structure because the hierarchy does not appear to hold up under varying environmental conditions (Duan and Surrette, 2007).

One way to try to quantify the effects of various environmental components is to examine multiple-species model systems. An et al. (2006) recently developed a dual-species model system using *Pseudomonas aeruginosa* and *Agrobacterium tumefaciens* to examine which bacterial factors might influence competitive ability in a multi-species biofilms. Their studies revealed that in both biofilms and defined culture medium *P. aeruginosa* consistently out-competed *A. tumefaciens*, primarily due to its faster growth

rate, although QS and motility were found to be important as well. Several different QS mutants were impaired in their competitive ability, likely due to the fact that many of the QS-regulated secreted products could be useful in nutrient acquisition, particularly the secretion of proteases that could degrade exoproducts of *A. tumefaciens* to use as additional carbon sources (Parsek and Greenberg, 2000). Another possibility is that these mutants are deficient in the production of QS-controlled toxins that could negatively impact *A. tumefaciens* growth. This approach, while still far removed from natural environmental conditions, has provided a means for identification of key features involved in microbial competition.

One environment where a multitude of bacteria co-inhabit is the rhizosphere, or zone immediately surrounding the roots of a plant. Roots exude many plant secondary metabolites that affect their interactions with soil-borne microbes (Bais et al, 2006). Rhizodeposition of these carbon-rich exudates can attract or inhibit the growth of various microorganisms, and can account for as much as 44% of the total photosynthetically fixed carbon of the plant (Grayston et al., 1998; Patterson and Sims, 2000). This carbon cost however, results in great benefits to the plant by maintaining microbial communities to recycle nutrients, produce growth-promoting hormones, offer protection from pathogenic microbes, and form symbiotic relationships that assist the plant in uptake and utilization of scarce environmental nutrients (Morgan et al., 2005). However, aside from the steady supply of carbon, the benefits or detriments of this rhizosphere effect on the competition between soil microbes is often overlooked. A few studies have shown that exudates are directly responsible for the diversity of rhizosphere microbial populations. For instance, one study demonstrated that *Acinetobacter* and *Chryseomonas spp.*, the

dominant microbial species in rhizospheres of clover, were largely replaced by *Aquaspirrillum spp.* that are typically found in the rhizosphere of *Gypsophila paniculata* (common Baby's breath) when saponins from *G. paniculata* were added to the rhizosphere of clover (Fons et al., 2003). Another study showed that by changing the plant species grown in a particular soil, diversity of native fungal populations dropped dramatically within three generations, and that activated carbon that absorbs root exudates eliminated this effect (Broeckling et al., 2008). These data indicate that plant root exudates may exert strong effects on microbial competition.

To examine the effect of root exudates on bacterial competition, we adapted the dual-species *P. aeruginosa-A. tumefaciens* model developed by An et al. (2006) to incorporate *Arabidopsis thaliana*, a model plant species that is both genetically tractable and whose root exudation under varying conditions has been studied extensively (Walker et al., 2003; Steeghs et al., 2004; Bais et al., 2005; Badri et al., 2008). Bacterial co-cultures were grown in liquid medium with *A. thaliana* plants and bacterial counts were obtained from the culture medium and the root surfaces. Additionally, both of these bacterial species are pathogenic to *A. thaliana* under the laboratory conditions used for these experiments, and therefore, the negative effects to the plant were measured in terms of plant mortality, and loss of total and root biomass. Finally, concurrent studies examining the changes in *A. thaliana* root exudate profiles in response to these bacteria alone and in co-culture are also being conducted to complement these studies.

MATERIALS AND METHODS

Plant Material and Bacterial Cultures

Seeds of *Arabidopsis thaliana* Col-0 were obtained from Lehle Seeds (Roundrock, TX). All seeds were surface sterilized and germinated on static Murishage and Skoog medium in growth chambers maintained at 25°C and programmed with a 16-hour light cycle. A *Pseudomonas aeruginosa* PAO1 wild-type pDSKeGFP with resistance to kanamycin at 500 μg ml^{-1} was provided by Dr. Li Fanyang (University of Singapore). A *P. aeruginosa lasR/rhlR* signal mutant carrying a gene conferring resistance to 100 μg ml^{-1} ampcillin and an *Agrobacterium tumefaciens* strain carrying a gene conferring resistance to 100 μg ml^{-1} gentamicin was provided by Dr. Matthew Parsek (University of Washington) (An et al., 2006). Overnight cultures for plant infection were grown in Luria-Bertani (LB) broth at 30°C (*A. tumefaciens*) and 37°C (*P. aeruginosa*). All cultures were maintained in 20% glycerol stocks at –80°C and cultured with antibiotics where appropriate.

Bacterial Co-culture with *A. thaliana*.

Seven-day old seedlings of *A. thaliana* were transferred to 6-well plates with 5 ml of liquid MS medium amended with 3% sucrose as a carbon source and placed on rotary shakers at ambient temperatures (between 25-30°C) equipped with 16-hour light cycles. Once plants were 18-days old, a time point previously determined to be optimal for root exudation in *A. thaliana* under these culture conditions (Dr. Victor Loyola-Vargas, personal communication) plants were inoculated with overnight bacterial cultures. Cultures of *A. tumefaciens* and *P. aeruginosa* were added to a final OD_{600}= 0.02 into the

A. thaliana culture medium in the following ratios: 1:0, 1:1, 0:1 *A. tumefaciens: P. aeruginosa.* Using this same experimental design, *A. tumefaciens* was also grown in co-culture with a *P. aeruginosa* QS receptor mutant. Plants and bacteria were co-cultured for 72 hours on rotary shakers under the same conditions as described for plants alone. After 72 hours, plants were removed and visually assessed for disease development and fresh weights were obtained. Individual roots were collected and washed with sterile H_2O to remove any unattached bacteria. Roots were then placed in Eppendorf tubes with 1ml 10 mM $MgSO_4$ and vortexed for 1 hour to remove attached bacteria. Additionally, 1 ml of the culture medium from each plant was removed. Root and medium colony forming units (CFUs) were assessed using the drop plate method (Herigstad et al., 2001) on solid LB medium containing the appropriate antibiotics. Each experiment was conducted twice with at least 5 replicates per experiment.

RESULTS

Coculture with *A. thaliana* Effects Competition Between *A. tumefaciens* and *P. aeruginosa*

We found that when the two bacterial species were grown alone with *A. thaliana*, *P. aeruginosa* wild-type had an advantage in growth (approximately 3.5x higher), as determined by bacterial CFUs after 72 hours in culture (Figure 5.1A). This is consistent with data from An et al. (2006) showing that *P. aeruginosa* PAO1 grew faster than *A. tumefacens* in media supplemented with a variety of carbon sources. However, when the two bacterial species were added at a 1:1 ratio to liquid medium with *A. thaliana*, they had shifted to a ~2:1 ratio after 72-hours in co-culture, with *A. tumefaciens* showing an

advantage in growth (Figure 5.1A). Interestingly, a PAO1 quorum-sensing mutant (*lasR/rhlR*) growing alone in medium with *A. thaliana* showed slower growth than *A. tumefaciens* under the same conditions. However, when these two bacteria were added together at a 1:1 ratio with *A. thaliana*, the PAO1 mutant outgrew *A. tumefaciens* by ~3:1 after 72 hours.

Both of these bacterial species are capable of colonizing the roots of plants so we also examined the viable population of bacteria on the root surface of *A. thaliana.* To evaluate this, we determined CFUs per gram of fresh weight of bacteria recovered from washed *A. thaliana* roots. The trend was identical to that observed for bacterial populations in the liquid medium. The PAO1 wild-type had the highest number of CFUs on the root surface, followed by approximately equal numbers of *A. tumefaciens* and the PAO1 mutant when inoculated into the growth medium alone (Figure 5.1B). Again, when the two wild-type bacteria were added in co-culture at a 1:1 ratio, *A. tumefaciens* recovered from the root surface showed a ~3:1 advantage in colonization compared to *P. aeruginosa.* By contrast, and most surprisingly, when *A. tumefaciens* was co-cultured with the PAO1 *lasR/rhlR* mutant, the amount of PAO1 *lasR/rhlR* recovered from roots was 12x greater than the amount of *A. tumefaciens* (Figure 5.1B).

Bacterial Coculture Decreases Negative Effects of *P. aeruginosa* on *A. thaliana*.

Agrobacterium tumefaciens is a plant pathogenic bacterium that causes crown gall disease and *P. aeruginosa,* while not an actual plant pathogen, causes disease in *A. thaliana* under laboratory conditions (Rahme et al., 1995) and can effectively infect roots of A. thaliana when grown together in liquid cuture (Walker et al., 2004). Thus both of

these bacterial species had negative effects on the growth and overall viability of *A. thaliana* when grown together or alone with the plant. Plants inoculated with *P. aeruginosa* showed chlorosis and wilting in addition to extremely reduced biomass and root fresh weight (Figure 5.2A and Figure 5.2B). Those inoculated with *A. tumefaciens* also had greatly reduced biomass (Figure 5.2A and Figure 5.2B) and appeared overall less fit than controls, although they did not display any uniform symptomology and none of them had visible tumors. Interestingly, plants that were co-inoculated with both bacteria appeared healthier and had significantly less reduction in biomass and root weight than those inoculated with *P. aeruginosa* alone, and were more similar in appearance and biomass to those inoculated with *A. tumefaciens* alone (Figure 5.2A and Figure 5.2B). The *P. aeruginosa* QS mutants alone had less negative impact on the root fresh weight than the other two pathogens, but this did not significantly impact the overall biomass of the plant, and the overall inhibition observed was similar to that seen with *A. tumefaciens* and the co-culture of the two wild-type bacteria. Co-culture of *A. tumefaciens* with the QS mutant of *P. aeruginosa* showed similar results as well.

Figure 5.1. Colony forming units of *P. aeruginosa, A. tumefaciens*, and a *P. aeruginosa* QS receptor double mutant grown with *A. thaliana* plants alone (1:0 ratio) and in co-culture (1:1 ratio) after 72 h in (A) liquid MS medium supplemented with 3% sucrose or (B) on the roots of *A. thaliana* plants. Error bars are +1 SE.

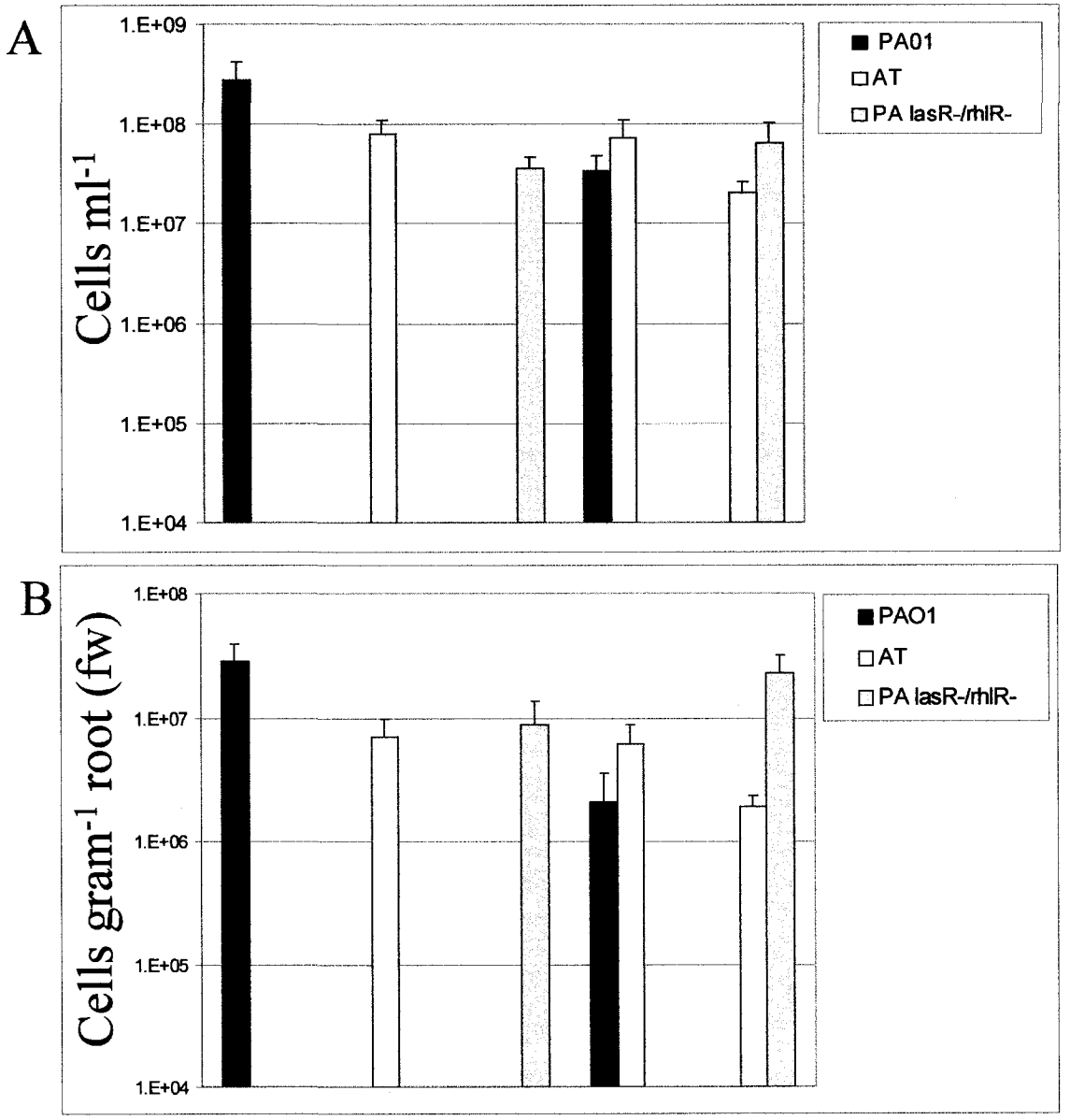
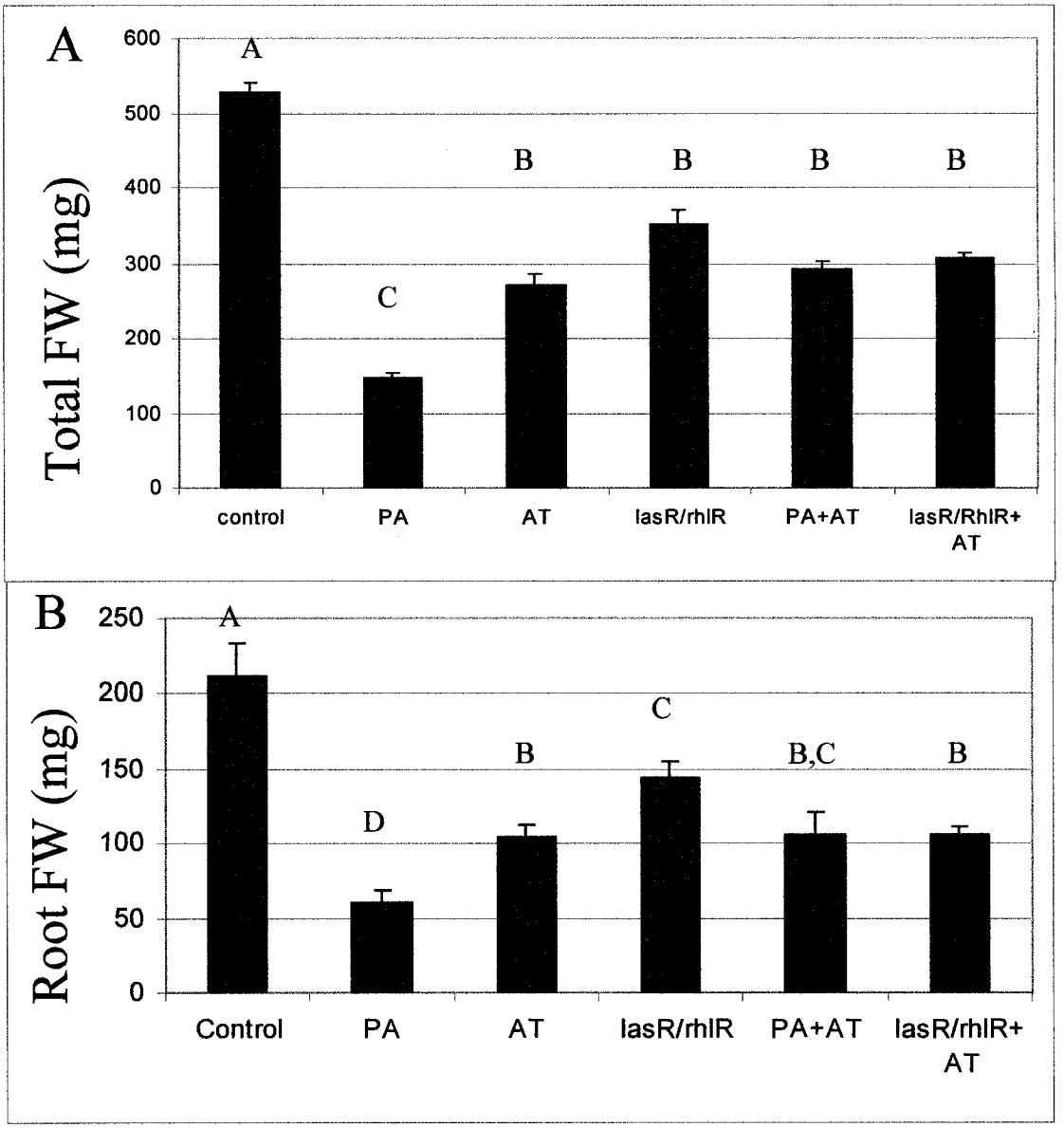


Figure 5.2. (A) Total fresh weight (FW) and (B) root FW of *A. thaliana* plants 72 after inoculation with *P. aeruginosa* wild-type and *lasR/rhlR* mutant and *A. tumefaciens* individually and in co-cultures. Error bars represent ± 1 SE. Bars with different letters denote significantly different means.



DISCUSSION

Although interesting, these results are very preliminary and introduce more questions than they answer. Before any valid interpretation of these data can be pursued it will be necessary to examine the growth curves of the two bacteria alone in MS medium, as this is not a medium optimized for bacterial growth and may result in different competitive outcomes than experiments previously conducted by An et al. (2006). However, the experiments performed thus far raise some interesting possibilities. In liquid co-cultures and biofilms, *P. aeruginosa* dominated *A. tumefaciens* (An et al., 2006); however, when *A. thaliana* was added to this equation *A. tumefaciens* apparently gained the competitive edge. Concurrent studies examining the root exudate profiles of *A. thaliana* inoculated with these two bacteria individually and in co-culture, show that the root exudate profiles vary with the different treatments, suggesting that there may be components in the root exudates that favor *A. tumefaciens* over *P. aeruginosa.* This could be in the form of anti-microbials that are only secreted in the presence of the two bacteria together, more efficient utilization of plant-secreted carbon sources by *A. tumefaciens*, or perhaps the activation of *A. tumefaciens* QS systems by opines that may be present in the root exudates of *A. thaliana.* Perhaps more interesting, is the fact that the QS receptor mutant of *P. aeruginosa* was more competitive than *A. tumefaciens* on both root surfaces and culture medium. A potential explanation for this is that the QS wild-type bacteria are secreting large amounts of AHLs to which the plant may be capable of detecting and responding. *Medicago truncatula* was shown to distinguish between bacterial AHLs and respond accordingly by altering root secretion profiles (Teplitski et al., 2000). The QS wild-type also secretes virulence factors that can elicit

root exudate responses from the plant and in turn may detriment the growth of the bacteria (Bais et al., 2005). A QS receptor mutant would be producing only the basal level of AHLs and would be deficient in many secreted exoproducts, perhaps preventing the plant from detecting its presence and therefore, allowing it to grow unchecked. However, none of these scenarios entirely account for the trends that we observed, and a detailed analysis of the exudates in addition to determination of growth rates in the MS medium should provide clues about which factors are important in these interactions.

The reduced negative effects of *P. aeruginosa* on the plant when grown in co-culture with *A. tumefaciens* suggest that under these conditions the pathogenicity of *P. aeruginosa* is reduced. As the QS mutant of *P. aeruginosa* also showed reduced negative effects on *A. thaliana* growth relative to *P. aeruginosa* wild-type, this might indicate that QS or the production of some QS controlled virulence factors are inhibited when the two wild-type bacteria are grown together with *A. thaliana.* Again an analysis of the components of the root exudates might clarify this point. Additionally assays to determine if virulence factor production is actually reduced in *P. aeruginosa* in co-culture and protein profiling of the exudates may also be useful. Finally, it should not be surprising that the plant growth effect and symptoms of the two wild-type bacteria in co-culture resemble infection by *A. tumefaciens* alone as *A. tumefaciens* was more efficient at colonizing the roots and may have acted to prevent *P. aeruginosa* from attaching and entering the roots. Detailed microscopic examination of the inoculated roots using fluorescence-tagged bacteria would enhance these data.

CHAPTER 6

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LIST OF ABBREVIATIONS

1. ADP- Adenosine Diphosphate
2. AHL: Acyl Homoserine Lactone
3. βGal: β-galactosidase
4. cDNA: Complementary Deoxyribonucleic Acid
5. CF: Cystic Fibrosis
6. CFU: Colony Forming Unit
7. DNA: Deoxyribonucleic Acid
8. FW: Fresh Weight
9. HPLC: High Performance Liquid Chromatography
10. HSD: Honestly Significant Difference
11. HSL: Homoserine Lactone
12. IVET: In Vivo Expression Technology
13. LRR: Leucine Rich Repeat
14. LSD: Least Significant Difference
15. MIC: Minimum Inhibitory Concentration
16. NMR: Nuclear Magnetic Resonance
17. NOD: Nucleotide-binding Oligomerization Domain
18. 4-NPO: 4-Nitro-Pyridine 1-Oxide

19. OD: Optical Density
20. PAMP: Pathogen Associated Molecular Pattern
21. PCR: Polymerase Chain Reaction
22. Pi: Inorganic Phosphate
23. PRR: Pathogen Recognition Receptors
24. qRT-PCR: Qualitative Real Time Polymerase Chain Reaction
25. QS: Quorum Sensing
26. QSI: Quorum Sensing Inhibitor
27. RNA: Ribonucleic Acid
28. ROS: Reactive Oxygen Species
29. RPM: Revolutions Per Minute
30. TLC: Thin Layer Chromatography
31. TTSS: Type Three (III) Secretion System

APPENDIX A

PAO1 genes significantly differentially regulated after in non-host relative to host interactions in *Nicotiana tabacum* cultivars Samsun and Xanthi.

Array element	Gene Function	Gene Name	Average Fold change	p-value
PA5180_at	conserved hypothetical protein	---	5.65	0.007
PA2120_at	hypothetical protein	---	5.12	0.002
PA3432_i_at	hypothetical protein	---	4.78	0.003
PA2691_at	conserved hypothetical protein	---	4.62	0.003
PA2204_at	probable binding protein component of ABC transporter	---	4.61	0.001
PA3092_fadH1_at	2,4-dienoyl-CoA reductase FadH1	*fadH1*	4.37	0.000
PA2663_at	hypothetical protein	---	4.22	0.011
PA0280_cysA_at	sulfate transport protein CysA	*cysA*	4.22	0.003
PA2575_at	hypothetical protein	---	4.08	0.029
PA0281_cysW_at	sulfate transport protein CysW	*cysW*	3.89	0.024
PA3431_at	conserved hypothetical protein	---	3.88	0.003
PA3584_glpD_at	glycerol-3-phosphate dehydrogenase	*glpD*	3.88	0.044
PA3450_at	probable antioxidant protein	---	3.75	0.002
PA3931_at	conserved hypothetical protein	---	3.73	0.013
PA1837_at	hypothetical protein	---	3.69	0.035
PA4442_cysN_at	ATP sulfurylase GTP-binding subunit/APS kinase	*cysN*	3.64	0.029
PA5181_at	probable oxidoreductase	---	3.61	0.029
PA5024_at	conserved hypothetical protein	---	3.60	0.004
PA3446_at	conserved hypothetical protein	---	3.44	0.007
PA2953_at	electron transfer flavoprotein-ubiquinone oxidoreductase	---	3.43	0.023
PA3933_at	probable choline transporter	---	3.37	0.034
PA1838_cysI_at	sulfite reductase	*cysI*	3.36	0.019
PA3444_at	conserved hypothetical protein	---	3.27	0.005
PA0830_at	hypothetical protein	---	3.20	0.010
PA0284_at	hypothetical protein	---	3.20	0.001

PA0283_sbp_at	sulfate-binding protein precursor	*sbp*	3.20	0.005
PA4195_at	probable binding protein component of ABC transporter	---	3.17	0.011
PA5105_hutC_at	histidine utilization repressor HutC	*hutC*	3.12	0.032
PA2382_lldA_at	L-lactate dehydrogenase	*lldA*	3.07	0.037
PA2263_at	probable 2-hydroxyacid dehydrogenase	---	3.07	0.040
PA5374_betI_at	transcriptional regulator BetI	*betI*	3.04	0.050
PA2321_at	gluconokinase	---	3.04	0.025
PA4443_cysD_at	ATP sulfurylase small subunit	*cysD*	3.02	0.040
PA0508_at	probable acyl-CoA dehydrogenase	---	3.01	0.016
PA4771_lldD_at	L-lactate dehydrogenase	*lldD*	2.87	0.026
PA4192_at	probable ATP-binding component of ABC transporter	---	2.85	0.022
PA0070_at	hypothetical protein	---	2.79	0.008
PA2173_at	hypothetical protein	---	2.76	0.042
PA0282_cysT_at	sulfate transport protein CysT	*cysT*	2.73	0.011
PA0201_at	hypothetical protein	---	2.65	0.011
PA3009_at	hypothetical protein	---	2.58	0.031
PA2323_at	probable glyceraldehyde-3-phosphate dehydrogenase	---	2.47	0.044
PA4193_at	probable permease of ABC transporter	---	2.42	0.019
PA3445_at	conserved hypothetical protein	---	2.36	0.036
PA2190_at	conserved hypothetical protein	---	2.35	0.032
PA1741_at	hypothetical protein	---	2.32	0.000
PA3183_zwf_at	glucose-6-phosphate 1-dehydrogenase	*zwf*	2.32	0.006
PA4047_ribA_at	GTP cyclohydrolase II	*ribA*	2.26	0.016
PA0837_slyD_at	peptidyl-prolyl cis-trans isomerase SlyD	*slyD*	2.22	0.046
PA3182_at	6-phosphogluconolactonase	*pgl*	2.18	0.028
PA2642_nuoG_at	NADH dehydrogenase I chain G	*nuoG*	2.14	0.028
PA0918_at	cytochrome b561	---	2.12	0.022
PA2634_at	probable isocitrate lyase	---	2.11	0.007
PA1831_at	hypothetical protein	---	2.11	0.030
PA4057_at	conserved hypothetical protein	---	2.10	0.053
PA3435_at	conserved hypothetical protein	---	2.09	0.031
PA2786_at	hypothetical protein	---	2.08	0.012
PA1051_at	probable transporter	---	2.06	0.007
PA4090_at	hypothetical protein	---	2.04	0.026
PA0033_at	hypothetical protein	---	-2.00	0.002
PA4397_panE_at	ketopantoate reductase	*panE*	-2.02	0.011
PA3593_at	probable acyl-CoA dehydrogenase	---	-2.02	0.037
PA5395_at	conserved hypothetical protein	---	-2.02	0.024
PA0239_at	hypothetical protein	---	-2.03	0.027
PA5396_at	hypothetical protein	---	-2.03	0.047

PA2873_at	hypothetical protein	---	-2.03	0.024
PA4236_katA_at	catalase	*katA*	-2.03	0.034
PA2052_cynS_at	cyanate lyase	*cynS*	-2.03	0.005
PA1991_at	probable iron-containing alcohol dehydrogenase	---	-2.03	0.012
PA2102_at	hypothetical protein	---	-2.04	0.043
PA0027_at	hypothetical protein	---	-2.04	0.036
PA3901_fecA_at	Fe(III) dicitrate transport protein FecA	*fecA*	-2.04	0.041
PA0271_at	hypothetical protein	---	-2.05	0.052
PA4986_at	probable oxidoreductase	---	-2.07	0.024
PA2675_at	probable type II secretion system protein	---	-2.07	0.024
PA1738_at	probable transcriptional regulator	---	-2.07	0.027
PA3886_at	hypothetical protein	---	-2.08	0.023
PA2694_at	probable thioredoxin	---	-2.08	0.012
PA5352_at	conserved hypothetical protein	---	-2.08	0.048
PA3622_rpoS_at	sigma factor RpoS	*rpoS*	-2.08	0.032
PA4908_at	hypothetical protein	---	-2.09	0.026
PA1502_gcl_at	glyoxylate carboligase	*gcl*	-2.12	0.036
PA5393_at	conserved hypothetical protein	---	-2.12	0.001
PA2097_at	probable flavin-binding monooxygenase	---	-2.14	0.042
PA1987_pqqC_at	pyrroloquinoline quinone biosynthesis protein C	*pqqC*	-2.15	0.014
PA4200_at	hypothetical protein	---	-2.16	0.028
PA4836_at	hypothetical protein	---	-2.16	0.011
PA0842_at	probable glycosyl transferase	---	-2.18	0.004
PA1371_at	hypothetical protein	---	-2.18	0.045
PA3869_at	hypothetical protein	---	-2.19	0.047
PA3292_at	hypothetical protein	---	-2.19	0.019
PA0207_at	probable transcriptional regulator	---	-2.20	0.052
PA5238_at	probable O-antigen acetylase	---	-2.21	0.045
PA2838_at	probable transcriptional regulator	---	-2.24	0.005
PA2373_at	conserved hypothetical protein	---	-2.24	0.052
PA0648_at	hypothetical protein	---	-2.25	0.002
PA2755_eco_at	ecotin precursor	*eco*	-2.26	0.037
PA0999_fabH1_at	3-oxoacyl-[acyl-carrier-protein] synthase III	*pqsD*	-2.26	0.048
PA0615_at	hypothetical protein	---	-2.26	0.037
PA0234_at	hypothetical protein	---	-2.26	0.005
PA1337_ansB_at	glutaminase-asparaginase	*ansB*	-2.26	0.054
PA4914_at	probable transcriptional regulator	---	-2.27	0.031
PA1148_toxA_at	exotoxin A precursor	*toxA*	-2.28	0.043
PA2676_at	probable type II secretion system protein	---	-2.28	0.012
PA4638_at	hypothetical protein	---	-2.28	0.023
PA1706_pcrV_at	type III secretion protein PcrV	*pcrV*	-2.28	0.046
PA1167_at	hypothetical protein	---	-2.29	0.028

PA2497_at	probable transcriptional regulator	---	-2.30	0.045
PA3946_at	probable two-component sensor	---	-2.30	0.043
PA0174_at	conserved hypothetical protein	---	-2.31	0.020
PA0848_at	probable alkyl hydroperoxide reductase	---	-2.32	0.031
PA0168_at	conserved hypothetical protein	---	-2.33	0.040
PA1365_at	probable siderophore receptor	---	-2.33	0.039
PA5360_phoB_at	two-component response regulator PhoB	*phoB*	-2.33	0.009
PA3774_at	probable acetylpolyamine aminohydrolase	---	-2.34	0.034
PA1412_at	hypothetical protein	---	-2.34	0.003
PA3510_at	hypothetical protein	---	-2.35	0.019
PA2635_at	hypothetical protein	---	-2.36	0.000
PA1700_at	conserved hypothetical protein in type III secretion	---	-2.36	0.047
PA2132_at	chaperone CupA5	*cupA5*	-2.37	0.054
PA3544_algE_at	Alginate production outer membrane protein AlgE precursor	*algE*	-2.37	0.046
PA1019_mucK_at	cis,cis-muconate transporter MucK	*mucK*	-2.37	0.042
PA3325_at	conserved hypothetical protein	---	-2.37	0.003
PA1497_at	probable transporter	---	-2.37	0.016
PA0257_at	hypothetical protein	---	-2.38	0.009
PA1149_at	hypothetical protein	---	-2.39	0.006
PA3375_at	probable ATP-binding component of ABC transporter	---	-2.39	0.028
PA0444_at	N-carbamoyl-beta-alanine amidohydrolase	---	-2.39	0.052
PA0347_glpQ_at	glycerophosphoryl diester phosphodiesterase, periplasmic	*glpQ*	-2.40	0.041
PA2053_cynT_at	carbonate dehydratase	*cynT*	-2.40	0.003
PA1566_at	conserved hypothetical protein	---	-2.40	0.012
PA0878_at	hypothetical protein	---	-2.41	0.034
PA2678_at	probable permease of ABC-2 transporter	---	-2.41	0.053
PA0730_at	probable transferase	---	-2.41	0.032
PA1873_at	hypothetical protein	---	-2.42	0.015
PA4104_at	conserved hypothetical protein	---	-2.42	0.048
PA2771_at	conserved hypothetical protein	---	-2.43	0.003
PA4073_at	probable aldehyde dehydrogenase	---	-2.44	0.033
PA5416_soxB_at	sarcosine oxidase beta subunit	*soxB*	-2.44	0.015
PA3947_at	probable two-component response regulator	---	-2.45	0.016
PA0710_gloA2_at	lactoylglutathione lyase	*gloA2*	-2.46	0.046
PA1327_at	probable protease	---	-2.46	0.006
PA3749_at	probable major facilitator superfamily (MFS) transporter	---	-2.46	0.036

PA1264_at	probable transcriptional regulator	---	-2.46	0.004
PA3542_at	alginate biosynthesis protein Alg44	*alg44*	-2.47	0.029
PA1936_at	hypothetical protein	---	-2.48	0.017
PA3927_at	probable transcriptional regulator	---	2.51	0.042
PA1696_pscO_at	translocation protein in type III secretion	*pscO*	-2.53	0.000
PA4021_at	probable transcriptional regulator	---	-2.55	0.040
PA3501_at	hypothetical protein	---	-2.55	0.050
PA4844_at	probable chemotaxis transducer	---	-2.56	0.047
PA0465_creD_at	inner membrane protein CreD	*creD*	-2.56	0.044
PA1910_at	probable tonB-dependent receptor protein	---	-2.57	0.033
PA2254_pvcA_at	pyoverdine biosynthesis protein PvcA	*pvcA*	-2.57	0.016
PA2342_mtlD_at	mannitol dehydrogenase	*mtlD*	-2.58	0.011
PA2425_at	PvdG	*pvdG*	-2.58	0.006
PA2074_at	hypothetical protein	---	-2.59	0.035
PA3035_at	probable glutathione S-transferase	---	-2.60	0.004
PA1311_phnX_at	2-phosphonoacetaldehyde hydrolase	*phnX*	-2.62	0.002
PA4625_at	hypothetical protein	---	-2.63	0.048
PA3849_at	conserved hypothetical protein	---	-2.64	0.002
PA3825_at	hypothetical protein	---	-2.64	0.045
PA1237_at	probable multidrug resistance efflux pump	---	-2.64	0.034
PA1945_at	probable transcriptional regulator	---	-2.66	0.039
PA1256_at	probable ATP-binding component of ABC transporter	---	-2.67	0.047
PA0634_at	hypothetical protein	---	-2.67	0.046
PA5540_at	hypothetical protein	---	-2.67	0.002
PA0694_exbD2_at	transport protein ExbD	*exbD2*	-2.68	0.008
PA0646_at	hypothetical protein	---	-2.69	0.005
PA0647_at	hypothetical protein	---	-2.69	0.019
PA2892_at	probable short-chain dehydrogenase	---	-2.69	0.043
PA1297_at	probable metal transporter	---	-2.70	0.026
PA1690_pscU_at	translocation protein in type III secretion	*pscU*	-2.71	0.004
PA1345_at	hypothetical protein	---	-2.72	0.021
PA0575_at	conserved hypothetical protein	---	-2.74	0.051
PA2116_at	conserved hypothetical protein	---	-2.75	0.039
PA3508_at	probable transcriptional regulator	---	-2.79	0.038
PA4859_at	probable permease of ABC transporter	---	-2.80	0.029

PA3324_at	probable short-chain dehydrogenase	---	-2.81	0.015
PA1330_at	probable short-chain dehydrogenase	---	-2.81	0.052
PA2431_at	hypothetical protein	---	-2.82	0.023
PA3924_at	probable medium-chain acyl-CoA ligase	---	-2.82	0.003
PA5469_at	conserved hypothetical protein	---	-2.82	0.054
PA1134_at	hypothetical protein	---	-2.83	0.004
PA2837_at	probable outer membrane protein precursor	---	-2.84	0.023
PA3500_at	conserved hypothetical protein	---	-2.85	0.019
PA0909_i_at	hypothetical protein	---	-2.86	0.030
PA4099_at	hypothetical protein	---	-2.88	0.012
PA2268_at	hypothetical protein	---	-2.88	0.017
PA5543_at	hypothetical protein	---	-2.88	0.042
PA0129_gabP_at	gamma-aminobutyrate permease	*gabP*	-2.90	0.053
PA1393_cysC_at	adenosine 5'-phosphosulfate (APS) kinase	*cysC*	-2.91	0.030
PA0866_aroP2_at	aromatic amino acid transport protein AroP2	*aroP2*	-2.92	0.053
PA3496_at	hypothetical protein	---	-2.94	0.024
PA2338_at	probable binding protein component of ABC maltose/mannitol transporter	---	-2.94	0.023
PA1352_at	conserved hypothetical protein	---	-2.95	0.013
PA0637_at	conserved hypothetical protein	---	-2.96	0.015
PA3502_at	hypothetical protein	---	-2.98	0.013
PA5411_at	probable ferredoxin	---	-2.98	0.013
PA3219_at	hypothetical protein	---	-2.99	0.008
PA3921_at	probable transcriptional regulator	---	-2.99	0.043
PA0847_at	hypothetical protein	---	-2.99	0.044
PA0699_at	probable peptidyl-prolyl cis-trans isomerase, PpiC-type	---	-3.00	0.053
PA2674_at	probable type II secretion system protein	---	-3.01	0.033
PA2188_at	probable alcohol dehydrogenase (Zn-dependent)	---	-3.01	0.014
PA0631_at	hypothetical protein	---	-3.03	0.033
PA5058_phaC2_at	poly(3-hydroxyalkanoic acid) synthase 2	*phaC2*	-3.04	0.055
PA3377_at	conserved hypothetical protein	---	-3.04	0.054
PA1499_at	conserved hypothetical protein	---	-3.05	0.042
PA4098_at	probable short-chain dehydrogenase	---	-3.06	0.047
PA1907_at	hypothetical protein	---	-3.06	0.032
PA0618_at	probable bacteriophage protein	---	-3.06	0.027
PA5353_glcF_at	glycolate oxidase subunit GlcF	*glcF*	-3.08	0.018
PA0636_at	hypothetical protein	---	-3.09	0.044
PA0640_at	probable bacteriophage protein	---	-3.10	0.028

PA3758_at	probable N-acetylglucosamine-6-phosphate deacetylase	---	-3.11	0.037
PA2881_at	probable two-component response regulator	---	-3.12	0.004
PA4802_at	hypothetical protein /// tRNA-Sec	---	-3.14	0.055
PA1418_at	probable sodium:solute symport protein	---	-3.14	0.016
PA0616_at	hypothetical protein	---	-3.14	0.013
PA4585_rtcA_at	RNA 3'-terminal phosphate cyclase	*rtcA*	-3.14	0.041
PA1231_at	conserved hypothetical protein	---	-3.15	0.022
PA1525_at	alkane-1-monooxygenase 2	*alkB2*	-3.16	0.023
PA0622_at	probable bacteriophage protein	---	-3.16	0.001
PA2340_at	probable binding-protein-dependent maltose/mannitol transport protein	---	-3.17	0.017
PA0630_at	hypothetical protein	---	-3.18	0.025
PA5417_soxD_at	sarcosine oxidase delta subunit	*soxD*	-3.18	0.024
PA4829_lpd3_at	dihydrolipoamide dehydrogenase 3	*lpd3*	-3.19	0.054
PA2059_at	probable permease of ABC transporter	---	-3.20	0.049
PA0632_at	hypothetical protein	---	-3.21	0.003
PA0623_at	probable bacteriophage protein	---	-3.21	0.004
PA2055_at	probable major facilitator superfamily (MFS) transporter	---	-3.22	0.022
PA2099_at	probable short-chain dehydrogenase	---	-3.23	0.025
PA0979_s_at	conserved hypothetical protein	---	-3.23	0.018
PA0679_at	hypothetical protein	---	-3.24	0.038
PA1694_pscQ_at	translocation protein in type III secretion	*pscQ*	-3.25	0.016
PA0693_exbB2_at	transport protein ExbB2	*exbB2*	-3.28	0.012
PA0635_at	hypothetical protein	---	-3.28	0.018
PA3596_at	probable methylated-DNA--protein-cysteine methyltransferase	---	-3.29	0.019
PA1351_at	probable sigma-70 factor, ECF subfamily	---	-3.29	0.012
PA1391_at	probable glycosyl transferase	---	-3.30	0.035
PA0621_at	conserved hypothetical protein	---	-3.31	0.004
PA2219_opdE_at	membrane protein OpdE	*opdE*	-3.32	0.045
PA4299_at	hypothetical protein	---	-3.32	0.053
PA2354_at	probable transcriptional regulator	---	-3.33	0.040
PA0638_at	probable bacteriophage protein	---	-3.33	0.022
PA0644_at	hypothetical protein	---	-3.33	0.003
PA0673_at	hypothetical protein	---	-3.34	0.054
PA3922_at	conserved hypothetical protein	---	-3.36	0.032
PA4298_at	hypothetical protein	---	-3.37	0.044
PA3923_at	hypothetical protein	---	-3.38	0.016

PA2428_at	hypothetical protein	---	-3.38	0.035
PA0645_at	hypothetical protein	---	-3.38	0.006
PA1259_at	hypothetical protein	---	-3.40	0.022
PA5541_at	dihydroorotase	*pyrQ*	-3.40	0.008
PA0136_at	probable ATP-binding component of ABC transporter	---	-3.40	0.052
PA1025_at	probable porin	---	-3.40	0.010
PA0633_at	hypothetical protein	---	-3.41	0.014
PA0625_at	hypothetical protein	---	-3.41	0.002
PA4350_at	conserved hypothetical protein	---	-3.41	0.015
PA2036_at	hypothetical protein	---	-3.42	0.027
PA2465_at	hypothetical protein	---	-3.43	0.034
PA0619_at	probable bacteriophage protein	---	-3.43	0.004
PA0624_at	hypothetical protein	---	-3.47	0.002
PA0250_at	conserved hypothetical protein	---	-3.47	0.007
PA3383_at	binding protein component of ABC phosphonate transporter	---	-3.48	0.022
PA2517_xylY_at	toluate 1,2-dioxygenase beta subunit	*xylY*	-3.48	0.046
PA0911_at	hypothetical protein	---	-3.51	0.032
PA4037_at	probable ATP-binding component of ABC transporter	---	-3.53	0.013
PA0641_at	probable bacteriophage protein	---	-3.55	0.030
PA0627_at	conserved hypothetical protein	---	-3.56	0.010
PA0614_at	hypothetical protein	---	-3.56	0.013
PA4910_at	probable ATP-binding component of ABC transporter	---	-3.59	0.010
PA0617_at	probable bacteriophage protein	---	-3.59	0.005
PA0188_at	hypothetical protein	---	-3.61	0.045
PA0629_at	conserved hypothetical protein	---	-3.63	0.014
PA0682_at	HxcX atypical pseudopilin	---	-3.64	0.043
PA4105_at	hypothetical protein	---	-3.65	0.021
PA5391_at	hypothetical protein	---	-3.69	0.016
PA2257_pvcD_at	pyoverdine biosynthesis protein PvcD	*pvcD*	-3.70	0.011
PA2313_at	hypothetical protein	---	-3.70	0.038
PA1394_at	hypothetical protein	---	-3.73	0.024
PA0620_at	probable bacteriophage protein	---	-3.74	0.001
PA0565_at	conserved hypothetical protein	---	-3.77	0.019
PA0626_at	hypothetical protein	---	-3.77	0.018
PA4039_at	hypothetical protein	---	-3.79	0.013
PA1571_at	hypothetical protein	---	-3.81	0.040
PA0692_at	hypothetical protein	---	-3.81	0.005
PA0273_at	probable major facilitator superfamily (MFS) transporter	---	-3.81	0.042
PA3586_at	probable hydrolase	---	-3.85	0.016
PA2228_at	hypothetical protein	---	-3.89	0.036
PA0166_at	probable transporter	---	-3.89	0.017
PA4107_at	hypothetical protein	---	-3.91	0.012
PA4613_katB_at	catalase	*katB*	-3.92	0.012
PA3253_at	probable permease of ABC transporter	---	-3.92	0.018

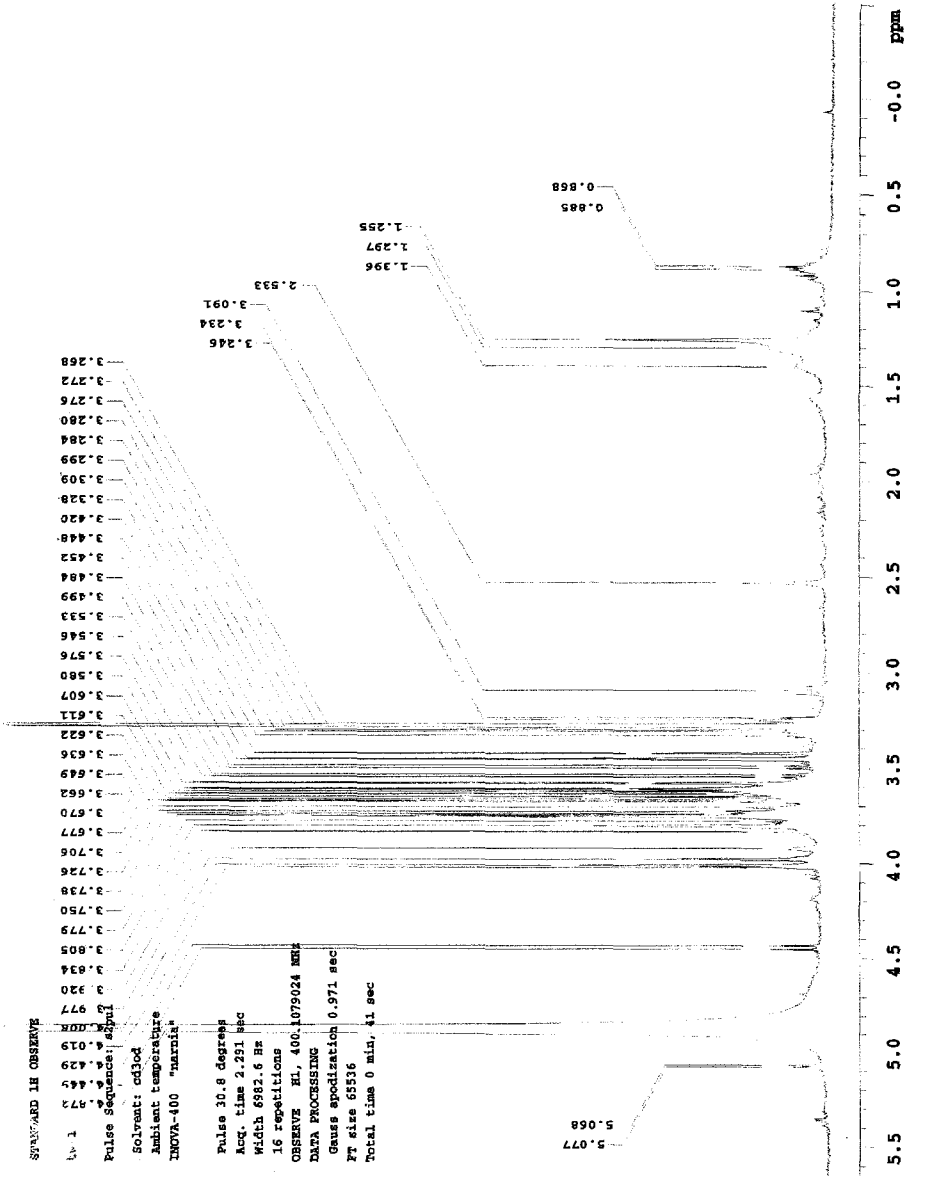
PA3296_phoA_at	alkaline phosphatase	*phoA*	-3.93	0.004
PA3063_at	hypothetical protein	*pelB*	-3.94	0.012
PA1785_at	conserved hypothetical protein	---	-3.96	0.033
PA3589_at	probable acyl-CoA thiolase	---	-3.96	0.023
PA0628_at	conserved hypothetical protein	---	-3.99	0.005
PA3287_at	conserved hypothetical protein	---	-4.09	0.010
PA0680_at	HxcV putative pseudopilin	---	-4.10	0.020
PA2060_at	probable permease of ABC transporter	---	-4.11	0.004
PA2129_at	chaperone CupA2	*cupA2*	-4.11	0.035
PA5468_at	probable citrate transporter	---	-4.20	0.030
PA2677_at	probable type II secretion protein	---	-4.27	0.001
PA4653_at	hypothetical protein	---	-4.33	0.034
PA4824_at	hypothetical protein	---	-4.36	0.049
PA0203_at	probable binding protein component of ABC transporter	---	-4.37	0.051
PA0639_at	conserved hypothetical protein	---	-4.39	0.004
PA4823_at	hypothetical protein	---	-4.42	0.014
PA4096_at	probable major facilitator superfamily (MFS) transporter	---	-4.42	0.012
PA5326_at	hypothetical protein	---	-4.42	0.005
PA1236_at	probable major facilitator superfamily (MFS) transporter	---	-4.42	0.005
PA3914_moeA1_at	molybdenum cofactor biosynthetic protein A1	*moeA1*	-4.46	0.022
PA2804_at	hypothetical protein	---	-4.50	0.054
PA1316_at	probable major facilitator superfamily (MFS) transporter	---	-4.54	0.000
PA4685_at	hypothetical protein	---	-4.55	0.046
PA3547_algL_at	poly(beta-d-mannuronate) lyase precursor AlgL	*algL*	-4.56	0.006
PA0978_s_at	conserved hypothetical protein	---	-4.57	0.030
PA0776_at	hypothetical protein	---	-4.58	0.039
PA4028_i_at	hypothetical protein	---	-4.64	0.007
PA4652_at	hypothetical protein	---	-4.74	0.011
PA3519_at	hypothetical protein	---	-4.76	0.052
PA1764_at	hypothetical protein	---	-4.80	0.022
PA0685_at	probable type II secretion system protein	---	-4.82	0.034
PA3671_at	probable permease of ABC transporter	---	-4.88	0.008
PA2346_at	conserved hypothetical protein	---	-4.90	0.005
PA0993_at	chaperone CupC2	*cupC2*	-4.92	0.033
PA1628_at	probable 3-hydroxyacyl-CoA dehydrogenase	---	-4.95	0.035
PA2922_at	probable hydrolase	---	-4.96	0.049
PA1743_at	hypothetical protein	---	-4.98	0.023
PA2093_at	probable sigma-70 factor, ECF subfamily	---	-4.99	0.022
PA5168_at	probable dicarboxylate transporter	---	-5.00	0.046

PA1699_at	conserved hypothetical protein in type III secretion	---	-5.00	0.041
PA5369_at	hypothetical protein /// 5S ribosomal RNA /// 23S ribosomal RNA /// tRNA-Ala /// tRNA-Ile /// 16S ribosomal RNA	---	-5.01	0.025
PA2113_at	probable porin	---	-5.03	0.003
PA2096_at	probable transcriptional regulator	---	-5.06	0.044
PA4903_at	probable major facilitator superfamily (MFS) transporter	---	-5.14	0.052
PA3384_phnC_at	ATP-binding component of ABC phosphonate transporter	*phnC*	-5.17	0.010
PA0443_at	probable transporter	---	-5.23	0.007
PA0493_at	probable biotin-requiring enzyme	---	-5.25	0.036
PA1598_at	conserved hypothetical protein	---	-5.26	0.027
PA4088_at	probable aminotransferase	---	-5.29	0.012
PA1569_at	probable major facilitator superfamily (MFS) transporter	---	-5.36	0.032
PA3319_plcN_at	non-hemolytic phospholipase C precursor	*plcN*	-5.37	0.036
PA0910_at	hypothetical protein	---	-5.37	0.033
PA0321_at	probable acetylpolyamine aminohydrolase	---	-5.57	0.023
PA4909_at	probable ATP-binding component of ABC transporter	---	-5.60	0.029
PA3062_at	hypothetical protein	*pelC*	-5.67	0.025
PA1416_at	conserved hypothetical protein	---	-5.68	0.041
PA1606_at	hypothetical protein	---	-5.72	0.024
PA2335_at	probable TonB-dependent receptor	---	-5.80	0.035
PA0698_at	hypothetical protein	---	-5.83	0.045
PA0688_at	probable binding protein component of ABC transporter	---	-5.83	0.004
PA0480_at	probable hydrolase	---	-5.84	0.051
PA4861_at	probable ATP-binding component of ABC transporter	---	-5.92	0.030
PA3376_at	probable ATP-binding component of ABC transporter	---	-5.98	0.043
PA0450_at	probable phosphate transporter		-6.03	0.011
PA0879_at	probable acyl-CoA dehydrogenase	---	-6.08	0.031
PA4540_at	hypothetical protein	---	-6.09	0.025
PA1254_at	probable dihydrodipicolinate synthetase	---	-6.12	0.048
PA3279_oprP_at	Phosphate-specific outer membrane porin OprP precursor	*oprP*	-6.24	0.043
PA4351_at	probable acyltransferase	---	-6.31	0.001
PA0977_at	hypothetical protein	---	-6.53	0.017
PA2215_at	hypothetical protein	---	-6.62	0.042

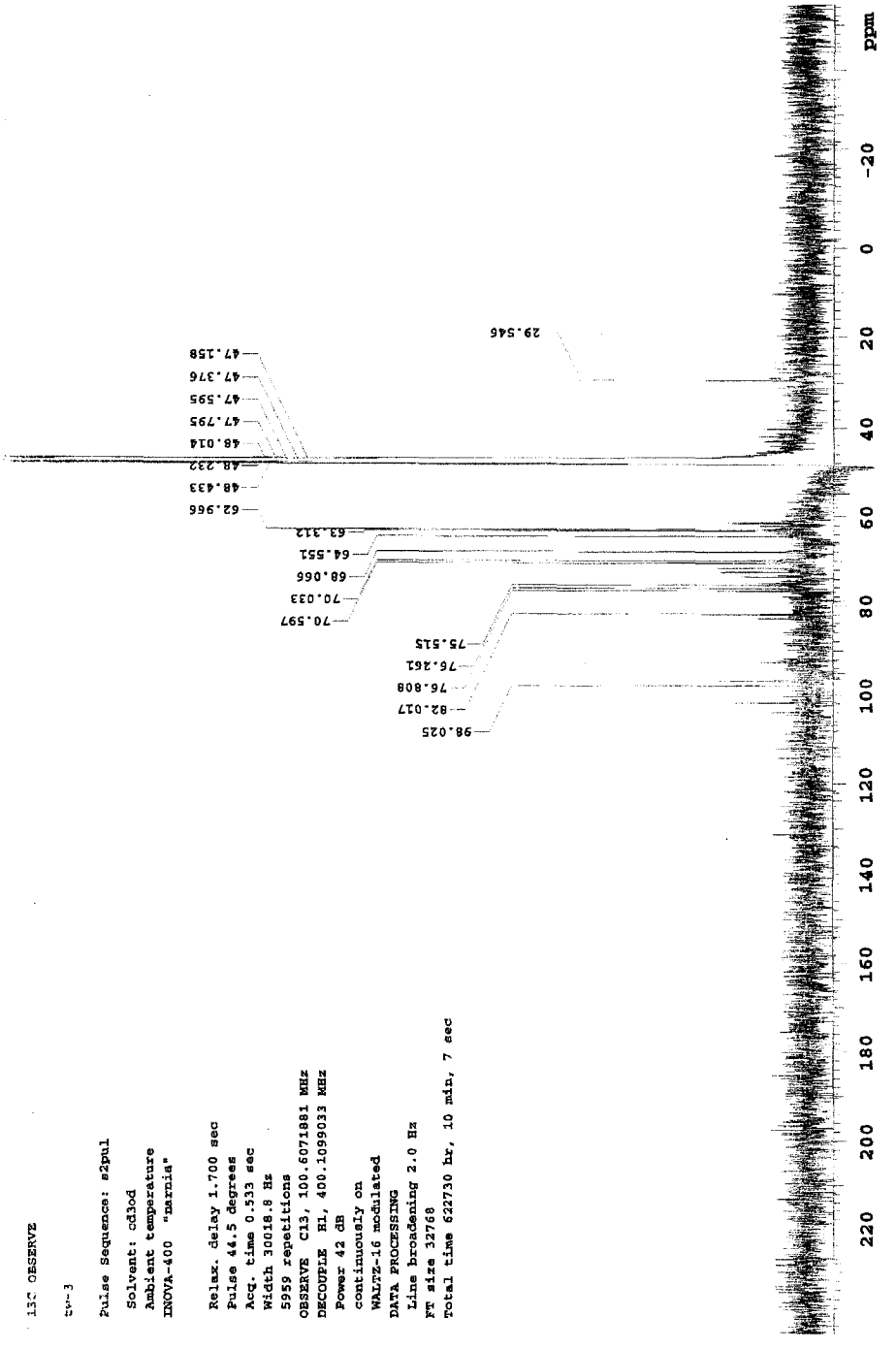
PA1646_at	probable chemotaxis transducer	---	-6.64	0.000
PA1267_at	hypothetical protein	---	-6.66	0.008
PA5384_at	probable lipolytic enzyme	---	-6.85	0.001
PA3280_oprO_at	Pyrophosphate-specific outer membrane porin OprO precursor	*oprO*	-7.07	0.000
PA4084_at	usher CupB3	*cupB3*	-7.11	0.000
PA1258_at	probable permease of ABC transporter	---	-7.17	0.030
PA3188_at	probable permease of ABC sugar transporter	---	-7.20	0.043
PA1797_at	hypothetical protein	---	-7.44	0.010
PA3237_at	hypothetical protein	---	-7.53	0.046
PA4167_at	probable oxidoreductase	---	-7.65	0.012
PA2217_at	probable aldehyde dehydrogenase	---	-7.82	0.035
PA1230_at	hypothetical protein	---	-7.86	0.004
PA4137_at	probable porin	---	-7.96	0.003

APPENDIX B

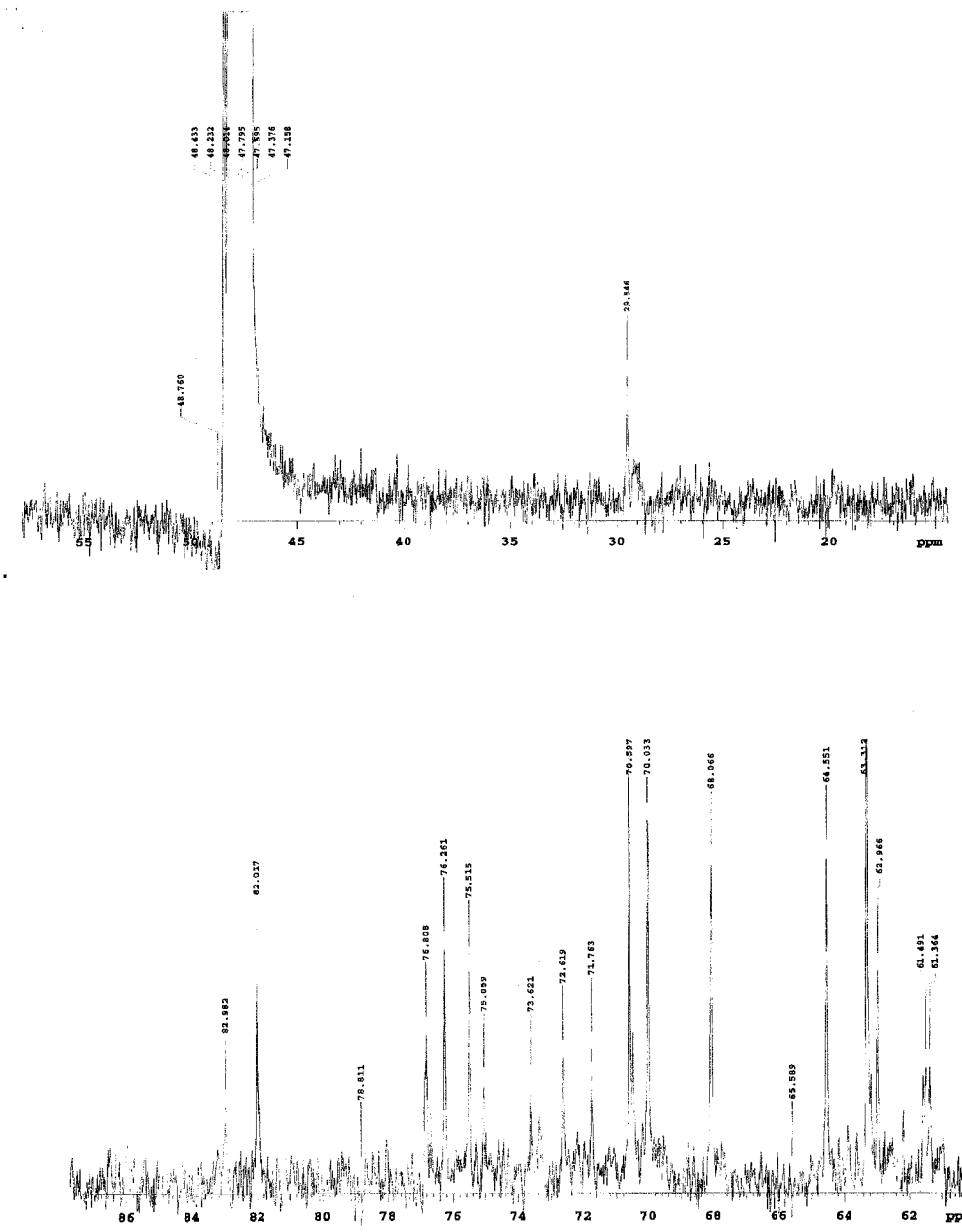
^{1}H NMR (400 mz) of *Pytolacca americana* crude root exudates.



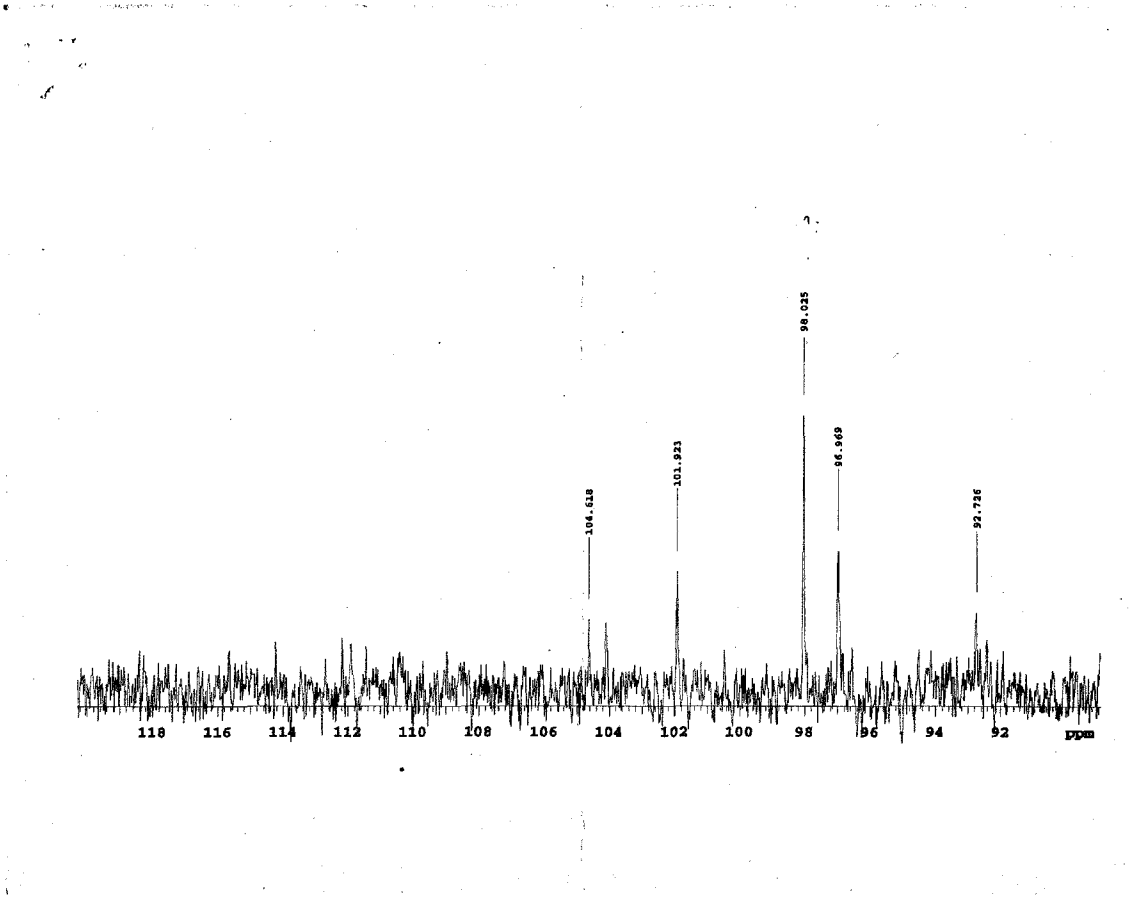
^{13}C NMR of *Phytolacca americana* crude root exudates.



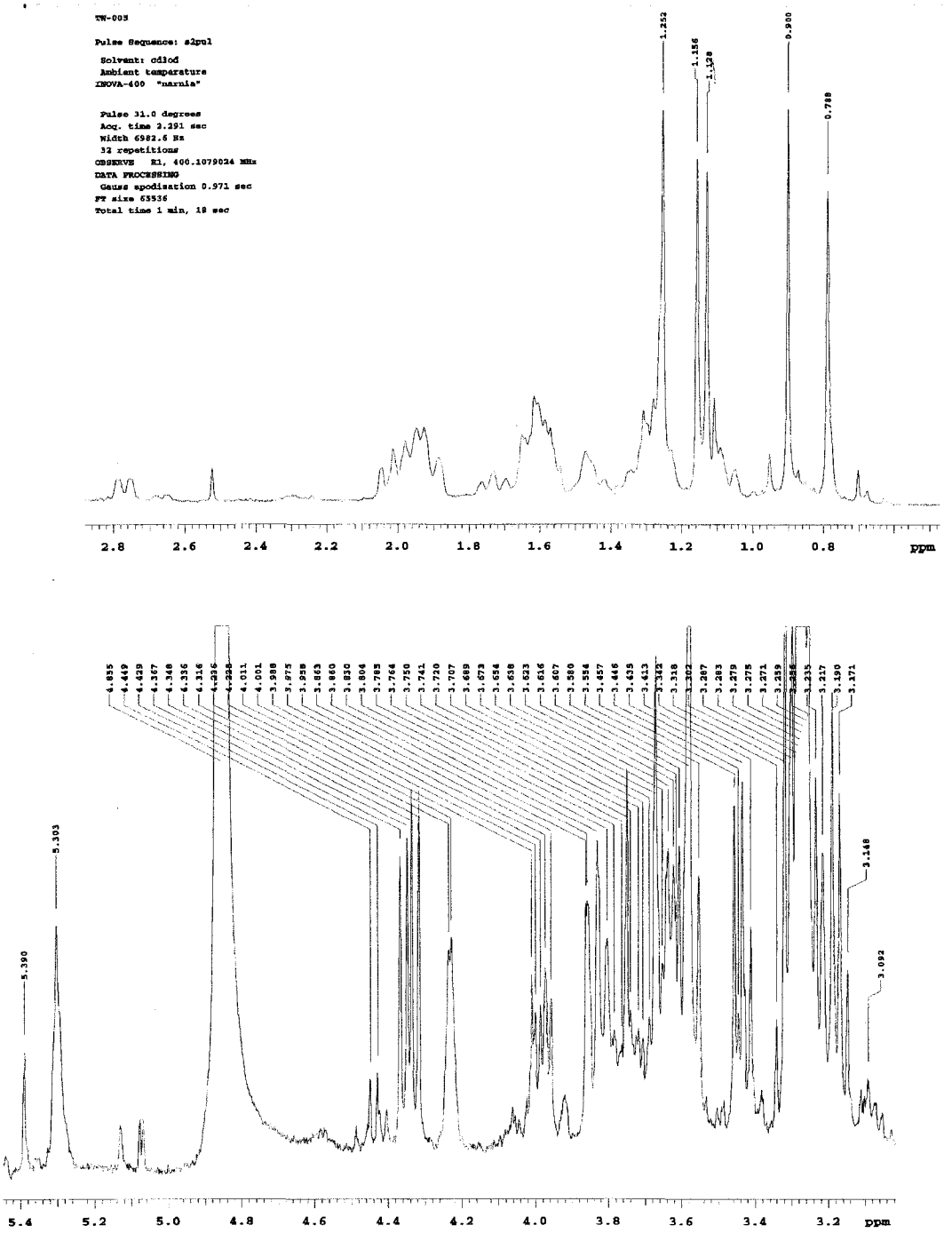
^{13}C NMR of crude *Phytolacca americana* root exudates.



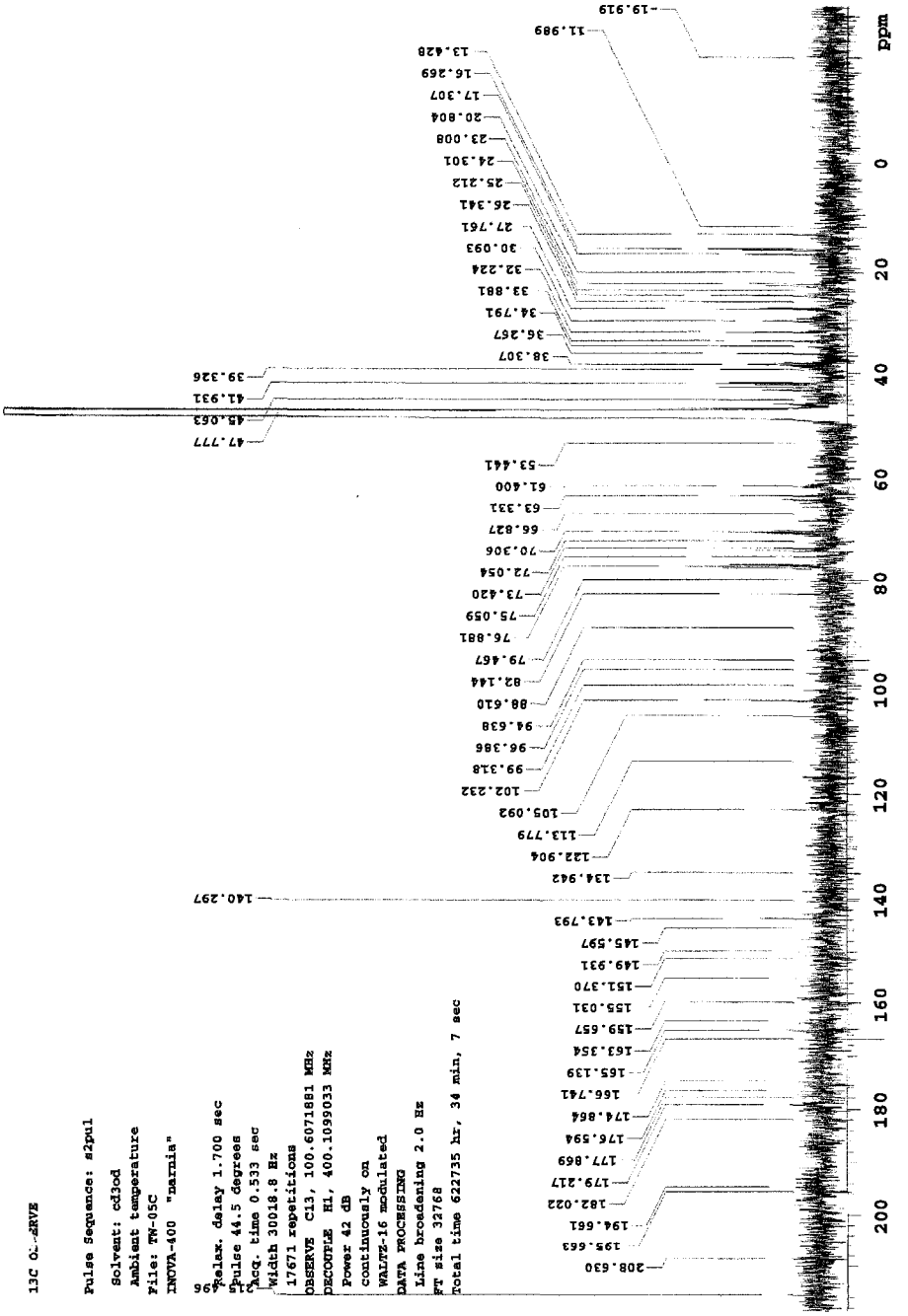
^{13}C NMR of *Phytolacca americana* crude root exudates.



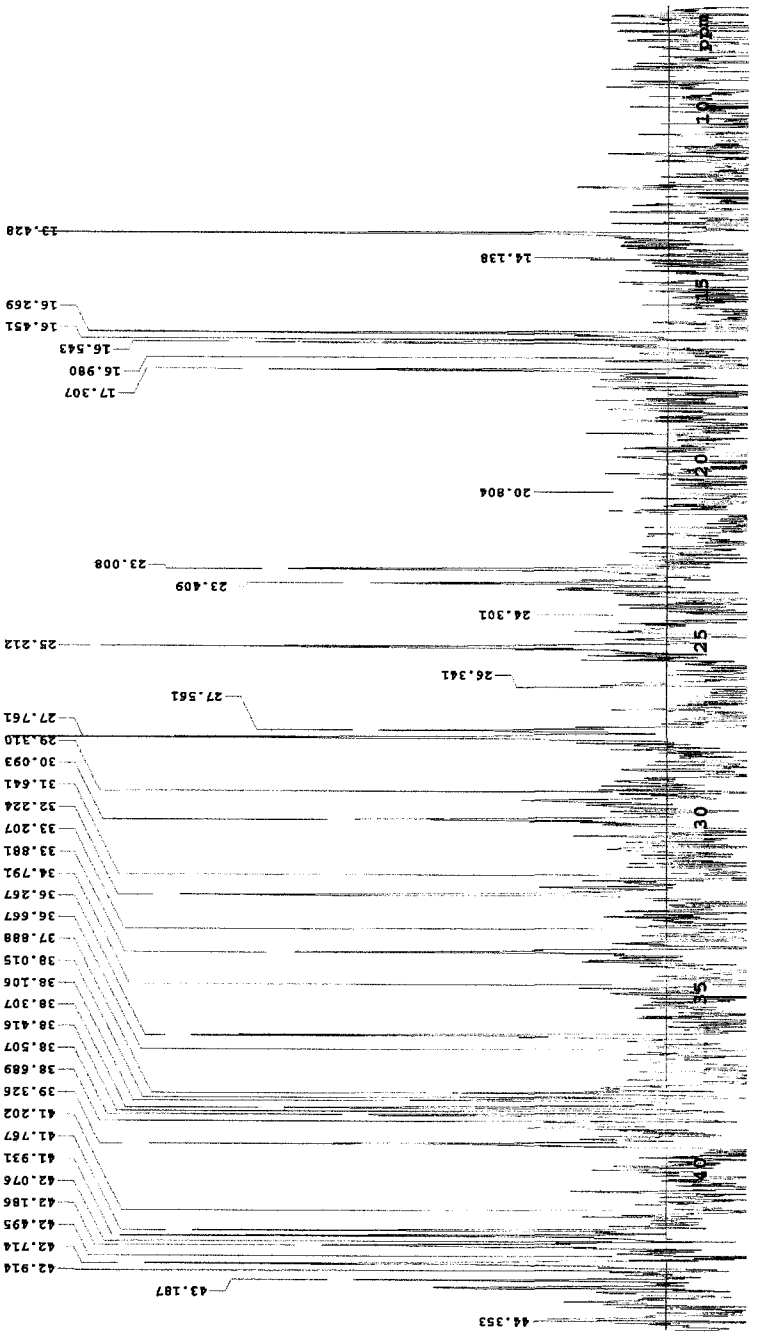
1H NMR of *Phytolacca americana* root extract fraction PAR3.16-3.20.



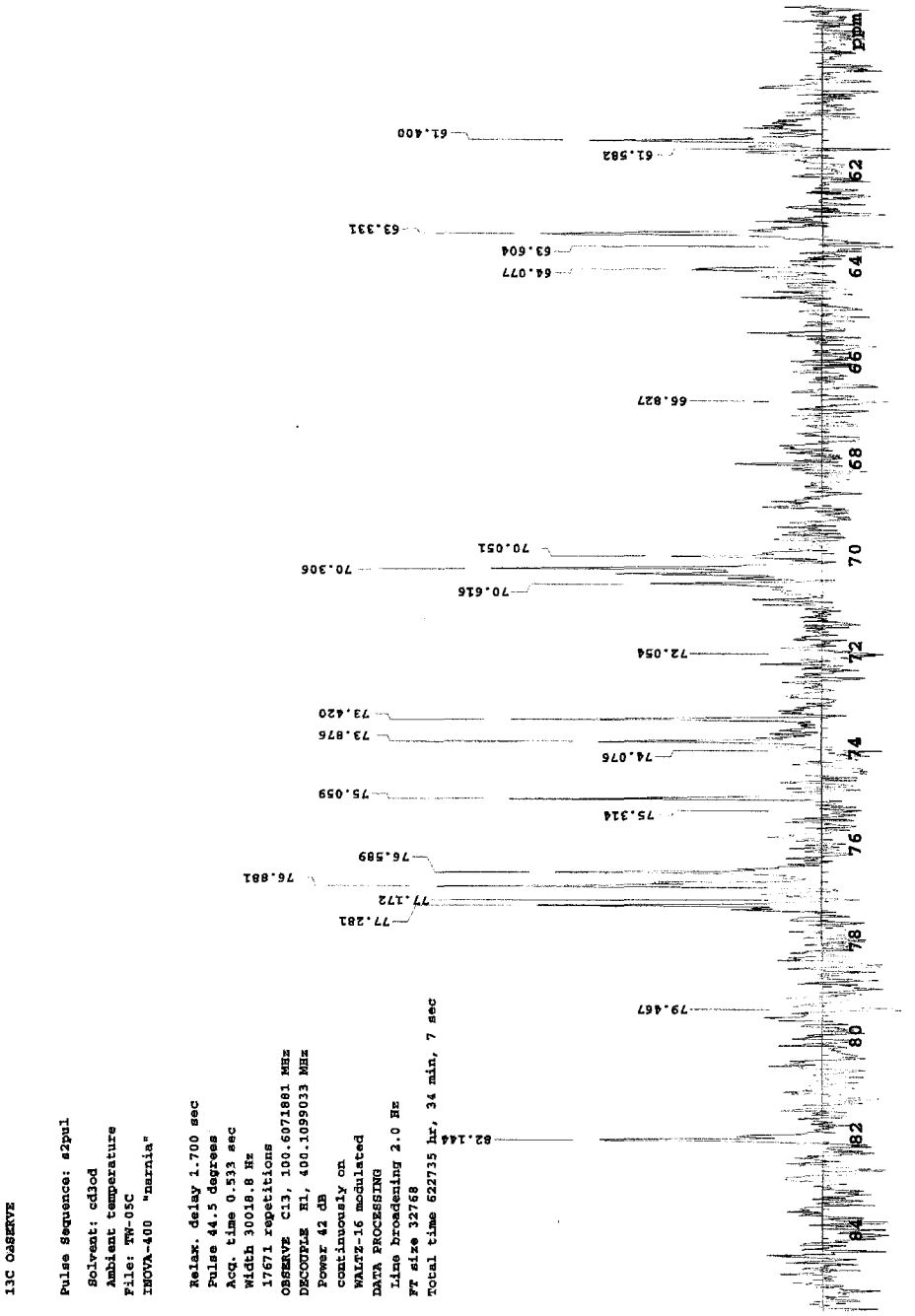
^{13}C NMR data for *Phytolacca americana* root extract fraction PAR 3.16-3.20.



^{13}C NMR data for *Phytolacca americana* root extract fraction PAR 3.16-3.20.



^{13}C NMR data for *Phytolacca americana* root extract fraction PAR 3.16-3.20.



^{13}C NMR data for *Phytolacca americana* root extract fraction PAR 3.16-3.20.