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# Role of Pseudomonas Produced Hydrogen Cyanide in Biological Control of Plant-parasitic Nematodes

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ROLE OF PSEUDOMONAS PRODUCED HYDROGEN CYANIDE IN  
BIOLOGICAL CONTROL OF PLANT-PARASITIC NEMATODES

**By**

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A DISSERTATION

Submitted to the Graduate School of the UNIVERSITY OF MISSOURI-ST. LOUIS In  
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DOCTOR OF PHILOSOPHY

in

BIOLOGY

with an emphasis in Cellular and Molecular Biology

St. Louis, August 2009

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## **Abstract**

Plant-parasitic nematodes are among the most devastating pests world-wide, causing extensive damage to important agriculture crops. The management of nematodes is challenging. Current methods used for nematode control rely mostly on using chemical nematicides and naturally resistant plants. Because of serious health and environmental problems associated with chemical control and over use of resistant plants resulting in resistant breaking strains of nematodes, alternative nematode-control methods are needed. Several potential nematode-biocontrol agents, including bacteria and fungi, have been studied for their antagonistic effect toward plant-parasitic nematodes. However, a concise picture about the biological agent's mechanisms used to antagonize nematodes remains elusive.

In the present study, over 60 different isolates of *Pseudomonas* were identified that were lethal to the free-living nematode, *Caenorhabditis elegans*. All nematode-lethal isolates of *Pseudomonas* were genetically, phenotypically, and biochemically characterized for their motility, exoprotease activity, and production of siderophores, polysaccharides, and secondary metabolites (cyanide, DAPG, PCA, pyrrolnitrin and pyoluteorin). These *Pseudomonas* strains were also screened for biocontrol activity against plant-parasitic nematodes (*Meloidogyne incognita* and *Heterodera glycines*), plant-pathogenic bacteria (*Agrobacterium rhizogenes*) and fungi (*Fusarium solani*). From our phenotypic characterization, one of the most common traits to most of the nematode-lethal strains was production of cyanide. Cyanide production was found in 87% of the *C. elegans*-lethal strains and 100% of the plant-parasitic nematode lethal strains.

One of the plant-parasitic nematode-lethal strains further characterized in this study was *Pseudomonas* sp. strain 15G2. The phenotypic characterization revealed that this strain is motile; a producer of exoproteases, siderophores, polysaccharides, and hydrogen cyanide (HCN); a colonizer of wheat and maize roots; and is lethal to both root-knot and soybean cyst nematodes. HCN produced by this strain is the major nematode lethal factor in *in vitro* and *in vivo* studies. From our experiments, two lines of evidence provide support for this conclusion. First, indirect exposure of nematodes to the wild-type 15G2 strain (where bacteria and nematode share the same air but do not come in contact with one another) induces nematode lethality while cyanide deficient 15G2 strains failed to kill. Second, application of wild-type 15G2 to soil produced a significant reduction in soybean cyst nematode population whereas the cyanide deficient mutant 15G2 strains had a reduced capacity for protecting plants against these plant-parasitic nematodes.

As further evidence for cyanide activity in the *in vivo* experiments we also demonstrate that cyanide production in soil can be monitored using cyanide-inducible gene expression in associated plant roots. The results from these studies provide significant insight into HCN as a mechanism by which *Pseudomonas* sp. 15G2 controls plant-parasitic nematodes and highlight the potential application of this strain to broaden the scope of options for nematode management.

## **DEDICATION**

For my wife Jodie and my parents

Aly and Tahany

Your patience, tolerance and understanding are immeasurable.

Mere words could never express the depth of my gratitude and love.

## **ACKNOWLEDGEMENTS**

I thank my supervisor, Dr. Christopher G. Taylor, for helping nurturing my growth as a scientist and as a person in the last four years. He has provided me with all resources and tools I needed to succeed and all the encouragements and enthusiasm to keep me going.

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# **CHAPTER I**

## **Introduction**

Plant-parasitic nematodes are among the most destructive pests that cause serious crop losses worldwide (Koenning et al., 1999). The management of nematodes is very difficult because they inhabit the soil and infect the underground parts of the plants and are not easily affected by soil treatments or agriculture practices (Galper et al., 1991; Kerry, 2000). Although different fumigants or nematicides have proven to be effective methods for controlling the nematode, they are no longer used because of serious safety and environmental issues. The search for the nematode's natural enemies has become the main focus of research to develop a successful biocontrol agent. Several microorganisms in soil infect or interfere with the nematode's life; using such microorganisms represents one of the possible solutions to control nematode populations. A large number of bacteria have been described in the literature as reducers of nematode populations. Among these bacteria are those from the *Pseudomonas* and *Bacillus* genera (Siddiqui and Mahmood, 1999). However, a clear or concise picture remains elusive about the diversity of mechanisms that these bacteria employ to reduce nematode populations. Here, we provide more insight about the diversity of lethal mechanisms used by the *Pseudomonas* genus, determine if these bacteria produce a common molecule(s) for antagonizing the nematode, and evaluate the practical role this molecule plays in nematode biocontrol.

## 1.1 Nematodes

Phylum Nematoda contains a complex blueprint of diversity and includes between 25,000 and 100 million known species (Dorris et al., 1999; Francois et al., 2006). Current classification methods rely on their morphological, physiological, and ecological diversity and place them onto two groups: free-living and parasitic nematodes (Dorris et al., 1999). Among the Nematoda phylum, most species are free-living in soil, freshwater, and marine systems. Only a relatively small number of species are parasitic to humans, animals and plants. The most common morphological traits used in the classification are buccal and pharyngeal structure. In addition, some of the anatomical structures such as cuticle, lip region, tail, intestine, and reproductive organs can also be used in nematode classification (Dorris et al., 1999; Francois et al., 2006). Recently, DNA molecular markers (such as rRNA genes), PCR-based assays, restriction digestions, genome sequences, and DNA microarrays have been used for nematode classification and construction of phylogenetic trees (Francois et al., 2006). Nematodes are microscopic roundworms that are considered to be one of the most abundant multicellular animals on earth (Baldwin et al., 2004). They evolved to dwell in diverse global and aquatic niches. While the free-living nematodes have no visible effects on humans, animals, and plants, the parasitic nematodes cause widespread problems.

### 1.1.1 *Caenorhabditis elegans*

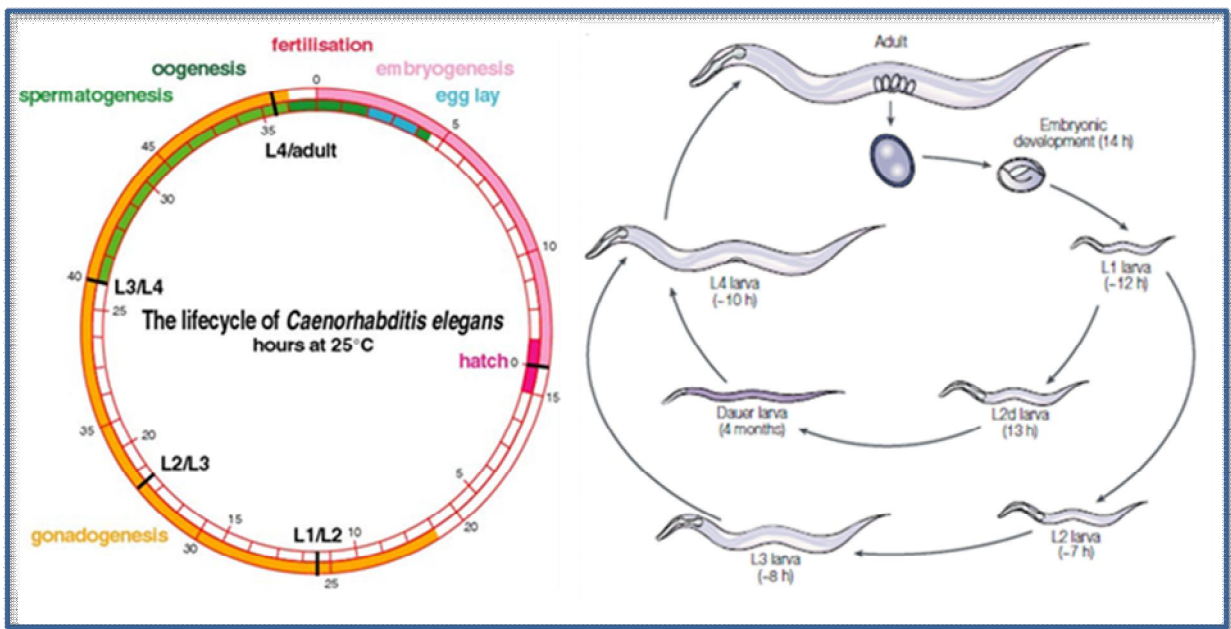
*Caenorhabditis elegans* is a free soil-living nematode that grazes on microbes and inhabits terrestrial and water niches. It is used extensively as a model organism to study developmental and functional biology of human diseases and disorders (Hashmi et al.,

2001; Bird et al., 2003; Kaletta and Hengartner, 2006). *C. elegans* possesses several features that make it a suitable animal model; they include a small size that makes it amenable for high-throughput bioassays, an ease of maintenance in the laboratory, a fast and convenient life cycle (3 days from egg to adult worm) without the requirement of a host (Figure 1.1), and the use of bacteria as a food source (Kaletta and Hengartner, 2006). In addition, the adult hermaphrodite of the *C. elegans* consists of 959 somatic cells that form many different organs and tissues. For example, the worm has muscles, nervous system, skin (hypodermis), gonad, reproductive systems and gastrointestinal tract that are similar in a drastically simplified way to many sophisticated animals (Jorgensen and Mango, 2002; Kaletta and Hengartner, 2006). The developmental simplicity of *C. elegans* permits the study of several biological processes at a single cell level. However, some studies cannot be performed in *C. elegans* due to the absence of corresponding tissue such as those related to the skeletal or immune system. At 25°C, the embryo goes through a series of cell divisions during the first five hours and after about 14 hours the L1 larva hatches from the eggshell. *C. elegans* has four larva stages (L1-L4) separated by periods of time. Under harsh conditions (such as starvation and elevated temperatures), the L1 enter the “dauer stage” and can resist the harsh conditions for months.

The morphological and developmental characteristics and nutritional requirements of *C. elegans* are similar to those of plant-parasitic nematodes, making it a convenient model for parasitism studies (Hashmi et al., 2001). Over 40 percent of the parasitic-nematode genes identified in nematode genome projects are found to share high similarity to the *C. elegans* genome (Hashmi et al., 2001). Such similarities make *C.*



*elegans* a good model organism to study these genes and reveal their role in the parasitism which, in turn, could lead to the development of new parasite management solutions.



**Figure 1.1.** The *C. elegans* life cycle. The graph on the left represents the timing of *C. elegans* developmental stages under favorable growth conditions (growing at 25°C). The diagram on the right shows an alternative developmental route (dauer stage) a nematode takes under unfavorable conditions such as crowded conditions and limited food. This dauer stage can resist the difficult conditions for months (Jorgensen and Mango, 2002).

### **1.1.2 Plant-parasitic nematodes.**

Plant-parasitic nematodes, ‘hidden enemies’, are obligate parasites (biotrophs) that cause significant damage to different agricultural crops. There are two different types of plant-parasitic nematodes: first, the migratory type, which include root-lesion nematodes that live outside the plant and feed on roots; and second, the sedentary type, which include the cyst (*Heterodera spp.*) and root-knot nematodes (*Meloidogyne spp.*).

#### **1.1.2.1 Migratory nematodes.**

Root-lesion nematodes have the ability to enter and migrate through root tissues while feeding on cellular components. In addition, they can move freely through the soil between feeding sessions (Smiley et al., 2005). They have broad host range and attack more than 350 different species of plants including corn, forage crops, tomato, potato, sugar beet, turf grasses, apple, cherry, conifers, roses and other ornamental plants. Both larval and adult forms enter young and mature plant roots and migrate through root tissue while feeding on cellular contents (Figure 1.2A). Symptoms caused by lesion nematodes include stunting and nutrient deficiencies in above-ground plant parts and the formation of deep necroses in the roots; these necroses can subsequently be colonized by pathogenic bacteria and fungi

#### **1.1.2.2 Sedentary nematodes.**

The soybean cyst nematode (*Heterodera glycines*) and root-knot nematode (*Meloidogyne spp.*) are sedentary parasites that are responsible for crop losses worldwide. Soybean cyst nematode (SCN) is a major pest of soybean (*Glycine max*) that represents a

limiting factor in soybean production. Under high nematode infestation, symptoms of infection with SCN include stunting and yellowing for the above-ground plant portions. The under-ground symptoms of SCN infection are determined by observing the adult females (cysts) on the roots (Hirrel, 1983). Root-knot nematodes (RKN) are responsible for approximately 50% of all nematode damage (Sasser and Krishnappa, 1980). They are capable of reproducing on over 2,000 different species of plants including most agricultural crops. Symptoms of infection by root-knot nematodes may include knot- or gall-like formations (Figure 1.2B) on the roots that inhibit the ability of the root to take up nutrients and water (Sijmons, 1993).

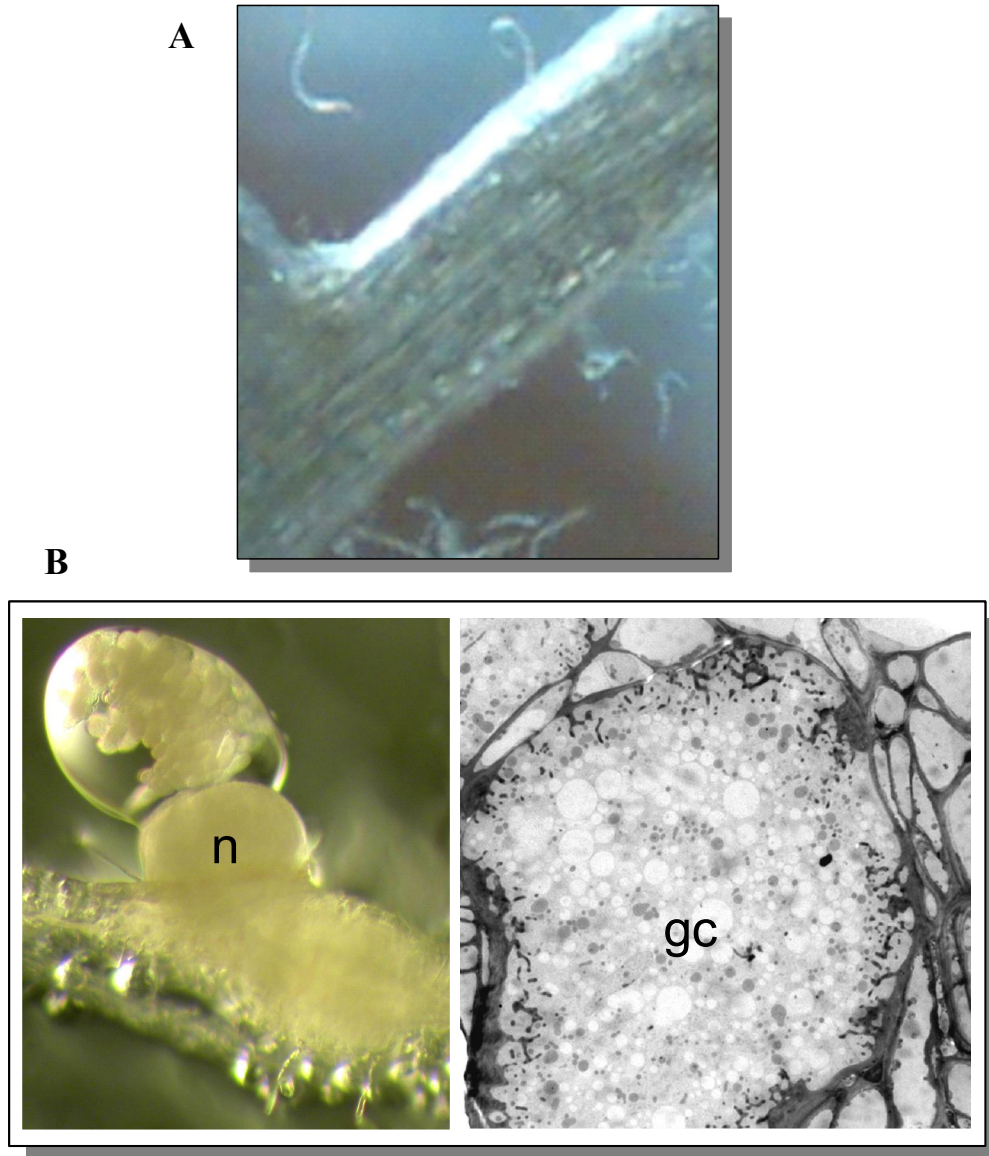
The sedentary nematodes, upon reaching their host plant, develop an intimate relationship with the roots, where they induce re-differentiation of root cells (feeding sites) and start feeding, reproducing, and become permanently attached to the roots of their host-plants. The induction of cell re-differentiation and feeding site formation (Figure 1.2B) are critical steps for the sedentary nematodes to obtain the nutrients required for growth and reproduction (Davis et al., 2004; Vanholme et al., 2004). The nematode's pathogenicity factors may play a direct role in the induction of syncytia (in cyst nematodes) or giant cells (in root-knot nematodes) formation (Vanholme et al., 2004).

Both root-knot and cyst nematodes are obligate parasites and feed on the cytoplasm of living plant cells to complete their lifecycle (Williamson and Gleason, 2003; Davis et al., 2004). These nematodes have sophisticated interactions with their host and they differ in their parasitic lifecycles (Figure 1.3). In soil, the eggs hatch as second stage motile juveniles (J2s) that migrate toward roots and then penetrate them,

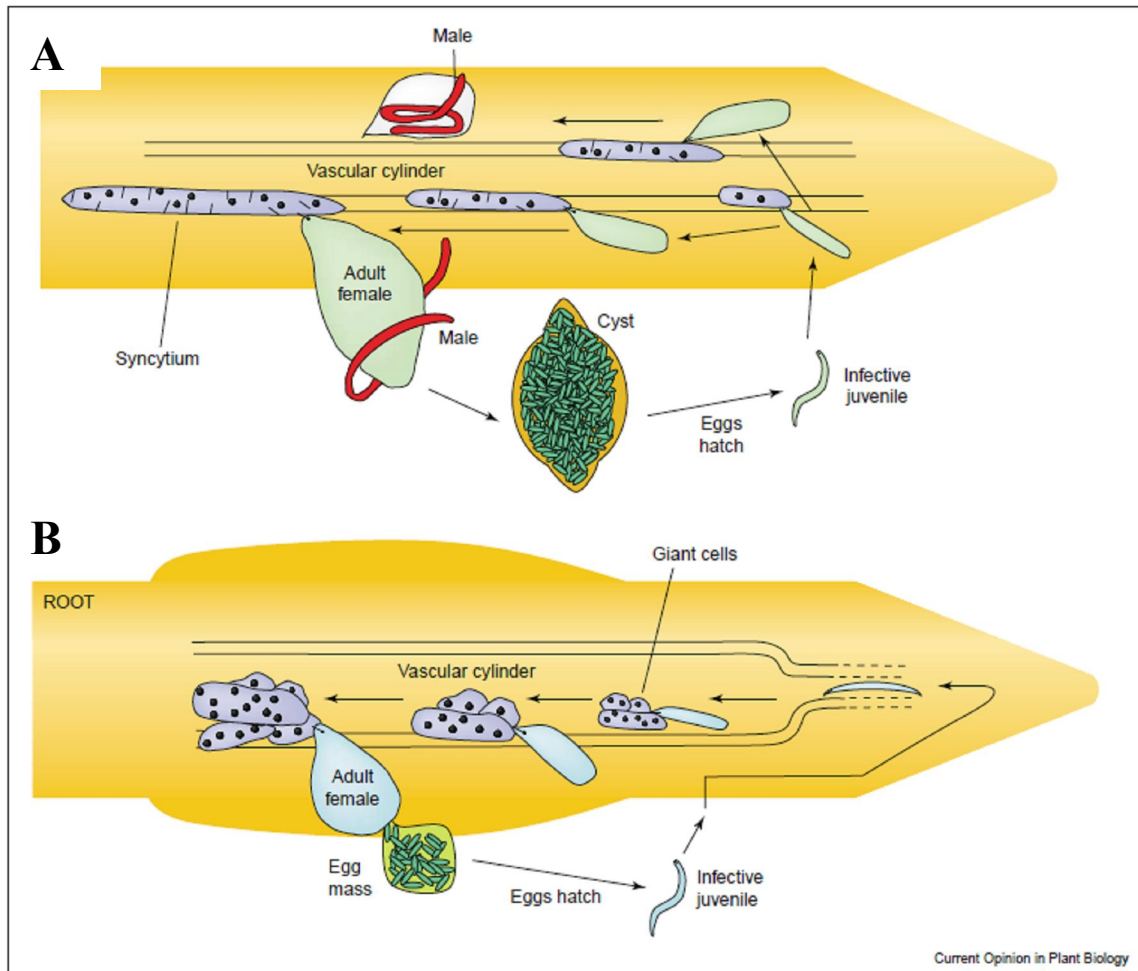
preferably behind the root cap in the zone of elongation or at rupture sites of lateral roots. Upon entering the roots, the J2s migrate intracellularly (cyst nematodes) or intercellularly (root-knot nematodes) to the vascular tissues and presumably release substances that then cause several physiological changes in root cells and the formation of feeding cells (Klink et al., 2007). These feeding cells serve as nutrient sinks that nematodes use for growth and subsequent parasite stages. In cyst nematodes, the initial feeding cell expands within the vascular cylinder by cell-wall disbanding followed by incorporation of adjacent cells and formation of a multinucleated state. Conversely, in root-knot nematodes cells at the feeding sites undergo sequential mitoses without cytoplasmic divisions followed by endoreduplication (additional DNA replication). These divisions lead to the formation of giant cells. The cells surrounding the giant cells will undergo asymmetric divisions causing swelling within the roots resulting in galls (Figure 1.2B).

The nematode juveniles develop and undergo three molts prior to the adult stage. In cyst nematodes, male nematodes feed for a few days to reach the end of J3 and become sedentary. The males then stop feeding and molt into the J4 stage that fertilizes females. However, the females continue to feed during the J3 and J4 stages and become lemon-shaped. After fertilization, the females die and become cysts containing eggs that will hatch under favorable conditions and complete the lifecycle (30 days). In root-knot nematodes, the females undergo a developmental transition after the last molt, which is characterized by a reduction in the somatic musculature (which renders the nematode immobile) and an increase in the size that turns the nematode pear-shaped. Within thirty days of infestation, the nematode reaches maturity as an adult female capable of

producing up to 1,000 eggs through mitotic parthenogenesis. However, the male nematodes eventually leave the roots and die.



**Fig. 1.2:** Plant-parasitic nematodes. **(A)** Root-lesion nematode (*Pratylenchus scribneri*) on soybean roots. **(B)** Root-knot nematode (n; *Meloidogyne incognita*) with developing egg mass on roots of *Arabidopsis thaliana* and gc; Root-knot nematode induced feeding site showing giant cells (provided by Dr. Christopher G. Taylor).



**Figure 1.3.** Representation of the plant-parasitic nematodes life cycles. (A) Soybean cyst nematode. (B) Root-knot nematode (Williamson and Gleason, 2003).

## 1.2 The impact of nematode on crops and possible control methods

Plant-parasitic nematodes are considered one of the most important pests that lead to crop losses worldwide. The cost of nematode management strategies used in the U.S. in 1982 was estimated to be US \$1 billion. In the Netherlands, between 1986 and 1990, the chemicals used for nematode control were three times more than the combined cost of chemicals used to combat fungi, insects, and weeds on experimental farms (Lewis et al., 1997). The plant-parasitic nematodes represent a continuing and serious problem that limits food production in developing countries (Koenning et al., 1999). For instance, in Africa, the plant-parasitic nematodes seriously threaten banana and plantain production (Li et al., 2007). One of the obstacles facing the estimation of yield loss due to nematode infection is the lack of clear, early symptoms. Frequently, the damages are considered underestimates as plants can often suffer approximately 35% yield loss without showing noticeable above-ground symptoms in field conditions. The growers cannot detect them until the infestation is severe (Atkinson, 1996). However, the damage to world crop production caused by plant-parasitic nematodes is estimated to be near \$100 billion annually (Koenning et al., 1999). The soybean cyst nematode is a serious problem for soybean crops in the United States. Damage due to soybean cyst nematodes has risen from \$2.6 billion worldwide (Sasser and Freckman, 1987) to over \$4 billion in the United States alone in 1994. In European Union countries, the combined annual yield losses due to sugar beet cyst nematode infection is estimated to be \$95 million (Lilley et al., 2005). The potato cyst nematode found in approximately 64% of potato fields is responsible for \$80 million yield losses annually (Bakker et al., 1993; Lilley et al., 2005).

Root-knot and root-lesion nematodes represent some of the most damaging forms of sedentary and migratory endoparasitic nematodes. Root-knot nematodes attack nearly every crop produced worldwide. Root-lesion nematodes attack some of the most important bioenergy crops produced in the United States, including corn and soybean. Numerous reviews and treatises have been written that thoroughly describe the damage to crops caused by root-knot nematodes (members of the *Meloidogyne* genus) (Sasser and Carter, 1985). They are among the most damaging nematodes in agriculture worldwide owing to their broad host ranges and global distribution. They cause an estimated crop loss of about \$100 billion annually (Khan et al., 2007). Cotton root-knot nematodes cause yield losses greater than any other cotton pathogens in the U.S. (Koenning et al., 2004).

Current methods of nematode control include crop rotation, application of chemicals, phytochemicals, use of genetically resistant varieties of plants, application of crop residues, and biological control. These methods have all been used to successfully control plant-parasitic nematodes but each method has limitations and drawbacks. Crop rotation by using non-hosts, poor hosts, or resistant hosts reduces the agronomic output a field can achieve while only delaying the ultimate buildup of nematodes. Resistant varieties of plants have been widely used for controlling nematodes. However, breeding for resistance is often complicated by the need for multiple genes (Williamson and Hussey, 1996). Chemical nematicides such as methyl bromide and aldicarb (Temik) have been effective in controlling nematodes but are rapidly being removed from the market because they are toxic to humans and animals and cause long-term contamination of ground water supplies. In recent years, a non-chemical alternative method for nematode



control was developed in which different crop residues are used as biofumigants (Cook and Baker, 1983; Hoitink and Fahy, 1986; D'Addabbo, 1995). These biofumigants introduce organic matter into soil, which in turn increase soil fertility and contribute to pathogen management and improve crop growth. For example, application of residues from pepper, tomato, cucumber, and strawberry crops combined with sheep manure significantly decreased the root-knot (*Meloidogyne incognita*) population in tomato (Piedrabuena et al., 2006).

Higher plants have yielded a broad spectrum of compounds (phytochemicals) that show antagonistic activities toward plant-parasitic and other nematodes. These phytochemicals include isothiocyanates, polythienyls, cyanogenic glycosides, glucosinolates, polyacetylenes, alkaloids, lipids, sesquiterpenoids, terpenoids, diterpenoids, quassinoids, steroids, triterpenoids, phenolics, and several other classes (Chitwood, 2002). Although these phytochemicals have tremendous potential to be used as a successful management strategy, their expensive costs limit their usage.

One potential alternative for plant-parasitic nematode management is the use of environmentally friendly biocontrol agents such as rhizobacteria. Biocontrol is the use of microorganisms that are able to suppress the growth or development of phytopathogens and their resulting diseases. Biocontrol has had some success in controlling nematodes but application is limited due to poor understanding about the mechanisms and conditions that biocontrol agents require to remain effective in the field (Zuckerman and Esnard, 1994). For example, some of the biological control agents have a relatively small range of activity compared to the use of nematicides (Baker, 1991; Raupach and Kloepper, 1998) and their performance in the field is often inconsistent. Understanding the

mechanisms and determining the factors that influence the biocontrol is the key to improving the reliability and efficiency of biocontrol systems. However, the most likely strategy for effective nematode control could be the combination of different technologies and techniques that will provide long-term integrated pest management.

### **1.3 Nematode biocontrol agents**

Soils that are naturally suppressive to soil-borne plant pathogens occur worldwide (Cook and Baker, 1983). In these soils indigenous microorganisms can effectively protect plants against different soil-borne pathogens. Conductive soils can be made suppressive by the addition of disease-suppressive microorganisms without disturbing the natural balance between beneficial and pathogenic microorganisms (Scher and Baker, 1980). In natural soil systems, nematodes and other microorganisms (fungi, bacteria, and mites) compete with each other for nutrients. These competitions include the production of metabolites that inhibit the growth of the competitors. This suggests that some microorganisms have developed mechanism(s) that allow them to survive under these conditions. For example, some root-associated bacteria, called rhizobacteria, have the ability to interfere with nematode growth and development, and use them as a nutritional source (Hanna and Tawfik, 1999; Siddiqui and Mahmood, 1999; Kerry, 2000). Identifying natural enemies of nematodes that interfere with their growth or development has become the main focus in developing nematode-biocontrol management.

Although biological control offers a sustainable means of reducing plant-parasitic nematodes, this approach has met with limited success in the context of modern cropping systems, possibly due to complex interactions between the nematode, its host, other rhizosphere inhabitants and the environment (Barker, 2003). Nevertheless, a large

number of bacteria have been described in the literature as reducers of nematode populations, including members of the genera *Agrobacterium*, *Alcaligenes*, *Bacillus*, *Clostridium*, *Desulfovibrio*, *Pasteuria*, *Pseudomonas*, *Serratia* and *Streptomyces* (Emmert and Handelsman, 1999; Siddiqui and Mahmood, 1999; Meyer, 2003). These bacteria are widely distributed and have broad spectrum activity. In addition, some of the bacteria with nematode antagonistic activities have been isolated from soils, hosts, and the nematodes themselves (Siddiqui and Mahmood, 1999; Kerry, 2000; Meyer, 2003). Members of the genus *Pasteruia* have been shown to infect both free-living and plant-parasitic nematodes and represent successful agents for the biocontrol of nematodes (Chen and Dickson, 1998). These bacteria form spores that can attach to the cuticles of a nematode's juveniles, germinate, and cause degeneration of the nematode reproductive system (Chen and Dickson, 1998).

Another group of the soil-microorganisms that can interfere and suppress the plant-parasitic nematodes is the nematophagous fungi. According to their mode of action, they can be grouped into three different categories: nematode-trapping fungi, parasitic fungi, and toxic fungi (Siddiqui and Mahmood, 1996). Extracellular hydrolytic enzymes produced by these fungi may account for their antagonistic mechanisms against plant-parasitic nematodes (Siddiqui and Mahmood, 1996). For example, species of the fungus *Trichoderma* have also been examined as a means of suppressing nematode populations (Barker, 2003; Siddiqui et al., 2003; Dong and Zhang, 2006). Both fungal and bacterial biocontrol agents for plant-parasitic nematodes are commercially available (Table 1.1) (Dong and Zhang, 2006).

Table 1.1 Commercially available biocontrol agents (Dong and Zhang, 2006)

Product name	Microbial origin and mode of antagonism	Company or institution	Country	Nematode target	Reference
Abamectin	<i>Streptomyces avermitilis</i> <sup>g, f</sup> Toxin production	Unspecified	Unspecified	<i>M. incognita</i>	El-Nagdi and Youssef (2004)
Biocon	<i>Paeclomyces litacinus</i> <sup>a, e</sup> Egg and female parasitism	Asiatic Technologies, Inc.	Manila, Philippines	Unspecified	Poinar and Georgis (1994)
Bionem	<i>Bacillus firmus</i> <sup>d, e</sup>	Unspecified	Israel	Root knot and other nematodes including <i>Heterodera avenae</i>	Wang and Liu (2004)
Deny	<i>Burkholderia cepacia</i> <sup>d, e</sup> Reduction of egg hatch and juvenile mobility	Stine Microbial Products	Madison, Wisconsin, U.S.A.	<i>M. incognita</i>	Meyer et al. (2001); Meyer and Roberts (2002)
DiTera	<i>Myrothecium</i> sp. <sup>a, f</sup> Toxin production	Valent Biosciences Corp.	Long Grove, Illinois, U.S.A.	<i>G. rostochiensis</i> , <i>G. pallida</i> , <i>H. glycines</i> , <i>H. schachtii</i> , <i>X. spp.</i> , <i>M. incognita</i> , <i>Radopholus</i> spp.	Twomey et al. (2000); Fernández et al. (2001)
Mixianning	<i>Paeclomyces litacinus</i> <sup>a, e</sup> Egg and female parasitism	Agricultural Institute, Yunnan Academy of Tobacco Science	Yunnan Province, P.R.China	Root-knot nematodes parasitizing tobacco	Zhu et al. (2001); Sun et al. (2002); Zhou and Mo (2002)
Nemout	Unspecified nematode-trapping fungus	Unspecified	Unspecified	<i>M. javanica</i>	Al-Hazmi et al. (1993) and Ibrahim (1994), as cited by Wang and Liu (2004)
Paecl (formerly Bioact) Royal 350	<i>Paeclomyces litacinus</i> <sup>a, e</sup> Egg and female parasitism	Prophyta	Philippines	Unspecified	Holland et al. (1999); Wang and Liu (2004); Mankau (1980); Wala and Vats (2000); Zhu et al. (2001); Zhou and Mo (2002)
Xianchongbike	<i>Arthrobotrys irregularis</i> <sup>a, e</sup> Nematode trapping <i>Pochonia chlamydosporium</i> <sup>a, e</sup> Mode of antagonism unknown	Unspecified Laboratory for Conservation and Utilization of Bio-resources, Yunnan University	France Yunnan Province, P.R.China	<i>M. spp.</i> Root-knot nematodes parasitising vegetable, tobacco, peanut, soybean and watermelon	

<sup>a</sup>Nematophagous fungus; <sup>b</sup>Endophytic fungus; <sup>c</sup>Actinomycete; <sup>d</sup>Bacterium; <sup>e</sup>Viable formulation; <sup>f</sup>Non-viable formulation (bionematicide-based)

In this chapter, only mechanisms used by the nematode-antagonizing *Pseudomonas* bacteria will be discussed.

#### **1.4 Mechanisms used by *Pseudomonas spp.* to control plant pathogens**

Recently, several studies have significantly provided new insights into the underlying mechanisms by which the biocontrol agents naturally function in their environment and antagonize other microorganisms. The genotypic and phenotypic characterizations of soil *Pseudomonas* have established this group of bacteria as potential biocontrol agents. They are abundant in arable soils and known to be excellent colonizers of the rhizosphere (Lugtenberg et al., 2001). They can use root exudates as a nutrient source, have a relatively high growth rate compared to other rhizobacteria, are easy to grow and maintain in the laboratory, and possess different mechanisms by which they antagonize different plant pathogens and interfere with their growth and development. Their activities against plant-pathogenic fungi and, to a lesser extent, nematodes have been documented (Gallagher and Manoil, 2001; Weller et al., 2002; Persello-Cartieaux et al., 2003; Haas and Defago, 2005). In addition, some non-pathogenic strains of *Pseudomonas* have the ability to trigger the plant systemic resistance against different phytopathogens (Chin et al., 2003). In the United States, a number of *Pseudomonas* inoculants used as biocontrol agents are commercially available (Table 1.2) (Mark et al., 2006).

The fluorescent *Pseudomonads* suppress phytopathogens by one or more mechanisms depending on the pathogen. These mechanisms can be grouped into five different categories: 1) competition for nutrients and niches, 2) predation, 3) induction of

plant-systemic resistance, 4) production of antimicrobial metabolites, and 5) direct stimulation of plant growth by increasing the nutrients, nitrogen fixation, and production of phytohormones (Thomashow and Weller, 1996; Weller et al., 2007).

#### 1.4.1 Competition for nutrients and niche exclusions

The ability of *Pseudomonas spp.* to rapidly establish themselves in the rhizosphere and compete with other microorganisms for the available nutrients represents one important mechanism for biocontrol. Junctions between root cells have been suggested to be the target niches for both *Pseudomonas* and other pathogens because root exudates leak from these sites (Chin-A-Woeng et al., 1997; Lagopodi et al., 2002; Bolwerk et al., 2003). The fast colonization of the roots by *Pseudomonas spp.* will prevent the pathogen's establishment on the root system and inhibit the infection. For example, *Pseudomonas fluorescens* WCS365 has a short generation time allowing it to rapidly colonize tomato roots (Chin-A-Woeng et al., 1997). In addition, competition for nutrients such as nitrogen, carbon, and iron play important roles in the biocontrol capabilities of *Pseudomonas spp.* In the rhizosphere, the soluble iron ( $\text{Fe}^{3+}$ ) is often limited. Iron is an important cofactor required for growth of all organisms. Under iron limitation, certain strains of *Pseudomonas* produce small molecules, called siderophores, which chelate and sequester iron in the rhizosphere, preventing its availability to other organisms (Thomashow and Weller, 1996; Haas and Defago, 2005). In addition, some other *Pseudomonas* strains such as *P. putida* are able to utilize siderophores (pseudobactins and pyoverdins) produced by other bacteria to increase their iron availability (Loper and Henkels, 1999). These pseudobactins and pyoverdins are diffusible pigments that give the Pseudomonads their fluorescence. *P. putida* deficient

in siderophore production is less suppressive to pathogens than the wild-type strain (Bakker et al., 1986). The ability of biocontrol *Pseudomonas* to produce siderophores or utilize siderophores produced by other bacteria gives them an advantage over pathogens by inhibiting the pathogen growth and leading to disease suppression.

#### **1.4.2 Antimicrobial metabolites**

Biological control by *Pseudomonas spp.* has been attributed in part to secondary metabolites, produced in the rhizosphere and in cultured bacteria that have antifungal action. Examples of these well-characterized compounds are: 2,4-diacetylphloroglucinol (DAPG), phenazine-1-carboxylic acid (PCA), phenazine-1-carboxamide (PCN, also known as chlororaphin), pyoluteorin (PLT), pyrrolnitrin (PRN), and hydrogen cyanide (HCN) (Figure 1.4). Most biocontrol *Pseudomonas* produce one or more of these secondary metabolites that account for their biocontrol activities. The activities of these compounds against plant pathogens are partially determined.

##### **1.4.2.1 Phenazines**

Phenazines (PCA and PCN) are among the more than 50 antimicrobial metabolites that are produced by *P. aeruginosa*, *P. chlororaphis (aureofaciens)*, *P. fluorescens*, and other genera of bacteria. They are similar to flavin coenzymes that inhibit electron transfer and damage lipids and other macromolecules (Haas and Defago, 2005). The most characterized phenazines are found to have broad spectrum activities against fungi, Gram-positive and Gram-negative bacteria (Laursen and Nielsen, 2004; Price-Whelan et al., 2006). For example, PCA and PCN produced by *P. chlororaphis* PCL1391 account for the suppression of both foot and root rot of tomato plants (Chin et al., 2000). In another example, phenazines have been shown to inhibit the hatching of

root-knot nematodes but do not kill juveniles (Kavitha et al., 2005). The “core” phenazine biosynthetic locus (*phz*) is well conserved (Pierson et al., 1995; Mavrodi et al., 2006), and PCR diagnosis for the *phzCD* junction sequence (McSpadden Gardener et al., 2001) is predictive of production. *PhzC* and *phzD* encode the first and third enzymes of the phenazine core pathway (Mavrodi et al., 2006).

#### 1.4.2.2 DAPG (2,4-Diacetylphloroglucinol)

DAPG is primarily produced by *P. fluorescens* and *P. putida*; it is possibly produced by additional species. This compound has antibacterial, antiviral, antifungal, and phytotoxic activities (Bangera and Thomashow, 1999; Haas and Keel, 2003). For example, 2,4-diacetylphloroglucinol produced by *P. fluorescens* strain F113 at high concentration is considered phytotoxic. It protects sugar beet from infection by *Pythium spp.* and potato plants from cyst nematode and soft rot infections (Cronin et al., 1997; Cronin et al., 1997). The compound is synthesized via the polyketide pathway, the enzymes of which are encoded by a 6.5 kb locus comprised of six genes (*phlABCDEF*) (Bangera and Thomashow, 1996; Mavrodi et al., 2001). This locus is conserved among the DAPG-producing *Pseudomonas spp.*, and DAPG production is correlated to the presence of this locus. Detection of the locus using PCR primers specific for *phlD* (Raaijmakers et al., 1997), the gene that encodes the polyketide synthase, is routine. Certain DAPG-producing *Pseudomonas* isolates exhibit preferential ability to colonize the roots of soybean and corn, the hosts of *P. scribneri* and *M. incognita*, respectively (McSpadden Gardener et al., 2005). Interestingly, this preference is not location-dependent (Mavrodi et al., 2001). Instead, the developmental stage of the host appears



to govern rhizosphere composition, possibly through fluxes in root exudates' quality and/or quantity (Picard et al., 2000).

#### 1.4.2.3 Pyoluteorin (PLT) and Pyrrolnitrin (PRN)

In the *P. fluorescens* isolate Pf-5, PLT synthesis involves a locus of ten genes (*pltABCDEFG*) that form the polyketide portion of the compound. The rest of the compound is comprised of a bichlorinated pyrrole adduct (Kraus and Loper, 1995; Nowak-Thompson et al., 1999). *PltB*, used in the PCR assays, encodes one of two polyketide synthases of the *plt* pathway. Genes encoding a PLT efflux transporter have recently been identified. Overexpression of the locus results in elevated PLT production (Huang et al., 2006). Production in the rhizosphere appears to be governed in part by the plant species (Kraus and Loper, 1995), suggesting that if PLT is nematocidal, rhizosphere colonization will play a role in nematode biocontrol. The mode of action for PLT has not yet been determined.

PRN was identified in *P. pyrrocinia* and is commonly produced by *P. fluorescens*, *P. chlororaphis* (*aureofaciens*) and other bacterial species (Hammer et al., 1997). The compound is derived from tryptophan by a biosynthetic pathway encoded by four genes (*prnABCD*). *prnC*, the target of the PCR assays, encodes the enzyme for the second chlorination step (Kirner et al., 1998). The antifungal activity and mode of action of PRN has been described (Tripathi and Gottlieb, 1969). PRN at growth inhibitory concentrations (10 mg/mL) target the terminal electron transport system and inhibit the respiration and growth of *Saccharomyces cerevisiae*, *Penicillium atrovirens*, and *P. oxalicum* (Tripathi and Gottlieb, 1969). *Pseudomonas* isolates frequently produce both PRN and PLT, or PRN in combination with DAPG (Maurhofer et al., 1995).

#### 1.4.2.4 Hydrogen Cyanide (HCN)

HCN production has been established in a small number of bacterial species such as *Pseudomonas* spp., *Rhizobium leguminosarum*, and *Chromobacterium violaceum* (Blumer and Haas, 2000). Cyanogenesis (production of HCN) has been extensively studied in *Pseudomonas* spp. It is widely generated by many isolates of *P. fluorescens* and *P. chlororaphis* under O<sub>2</sub>-limiting conditions. The highest HCN production occurs during the transition between the exponential and stationary phases (Castric et al., 1972). HCN production is influenced by several environmental conditions such as iron, phosphate, and oxygen availabilities (Blumer and Haas, 2000). In *Pseudomonas* spp., the structural genes *hcnA*, *hcnB*, and *hcnC* most likely form an operon that encodes for HCN synthases. The amino acid sequences of these genes share similarities with known dehydrogenases or oxidases. The *hcnB* and *hcnC* amino acid sequences contain a distinctive FAD- or NAD binding motif, ADP-binding motif in their N-terminal, and two transmembrane segments, which suggest that they are membrane-bound proteins (Ramette et al., 2006). The predicted polypeptides from HcnB (50 kDa) and HcnC (45 kDa) share similarities with the flavoenzyme, glycine oxidase. This may suggest that these HCN synthases catalyze the formation of HCN and CO<sub>2</sub> from glycine. It has been suggested that the *hcnA* sequence serves as a regulatory sequence for *hcnB* and *hcnC* (Blumer and Haas, 2000). Under O<sub>2</sub>-limitation, HCN synthase transfers four electrons to the respiratory chain and NO<sup>3+</sup> and NO<sup>2+</sup> serve as terminal electron acceptors for ATP synthesis. In contrast, in the presence of O<sub>2</sub>, the HCN synthase becomes rapidly inactivated. To date, little information is available about the mechanism of HCN production and different pathways have been suggested (Blumer and Haas, 2000). As a

respiratory inhibitor, HCN is expected to have broad-spectrum rhizosphere toxicity. Its nematicidal effects against *C. elegans* have been documented (Gallagher and Manoil, 2001).

The mechanism by which bacterial cyanide kills the nematode remains unexplored, but two possibilities have been suggested (Gallagher and Manoil, 2001). First, HCN causes a shortage of available oxygen (hypoxia), inhibits cytochrome oxidase and represses the adaptive response to the O<sub>2</sub>-limiting conditions. Second, HCN inhibition leads to accumulation of reactive oxygen species which, in turn, can cause significant damage to cell structures and lead to the nematode's death.

#### **1.4.2.5 Exoprotease production**

Some *Pseudomonas* strains produce extracellular enzymes that degrade the cell wall of phytopathogens resulting in damage and death of the pathogens. For example, *P. stutzeri* strain YPL-1 secretes extracellular chitinase and liminarinase that digest and degrade the mycelium of the fungus, *Fusarium solani* (Lim et al., 1991). Also, it has been demonstrated that the extracellular protease from *P. fluorescens* (Siddiqui et al. 2005), is generally considered to exert nematicidal activity by degradation of the nematodes cuticle (Siddiqui and Mahmood 1999). The resulting degradation products can be used by biocontrol *Pseudomonas* to support their growth.

#### **1.4.2.6 Other compounds**

Candidate inhibitors are not restricted to the compounds listed above; *Pseudomonas* isolates produce other types of compounds known to be inhibitory to eukaryotes. Among these are N-mercapto-4-formylcarbostyryl, a new class of secondary

metabolite from *P. fluorescens* G308 isolated from barley leaves (Fakhouri et al., 2001), rhamnolipid surfactants (Gunther et al., 2005), liposurfactants (Raaijmakers et al., 1997), and extracellular proteases (Siddiqui et al., 2005).

### 1.4.3 Induction of systemic resistance

Plants respond to pathogen infection by inducing their immune defense system, which is known as systemic acquired resistance (SAR) (Durrant and Dong, 2004). This system is characterized by the accumulation of salicylic acid (SA) and expression of pathogenesis related (PR) proteins (Durrant and Dong, 2004). Some non-pathogenic bacteria such as *Pseudomonas* spp. are capable of triggering a different immune response known as induced systemic resistance (ISR). Although ISR confers resistance against plant pathogens, it does not induce the accumulating SA or activate the PR gene expression. However, ISR is characterized by production of jasmonic acid and ethylene (Bakker et al., 2007). The presence of *Pseudomonas* in the rhizosphere protects the above-ground parts of the plant from infection by phytopathogens. For example, *P. fluorescens* strain WCS417 induces the ISR in *Arabidopsis thaliana* and protects it from infection by *P. syringae* pv. tomato (Pieterse et al., 1996). The *Pseudomonas* signals that trigger ISR have not been completely revealed. However, several studies have shown that some *Pseudomonas* spp. surface structures (such as flagella and lipopolysaccharides) or secreted metabolites (such as siderophores and antibiotics) play important roles in ISR induction (Bakker et al., 2007).

Table 1.2 USA-Commercially available *Pseudomonas* biocontrol inoculants (Mark et al., 2006).

Product name	Biocontrol organism	Target pathogen	Crop	Formulation/application	Manufacturer
Biolect Spot-Less ☉	<i>Pseudomonas aureofaciens</i> Tx-1	<i>Sclerotinia homeocarpa</i> (Dollar spot)	Turf	Liquid applied as spray	Eco Soil Systems Inc. (San Diego, CA)
EPA registered 1999		<i>Collectotrichum graminicola</i> (Anthracnose)			www.ecosoil.com
		<i>Pythium aphanidermatum</i> ( <i>Pythium</i> )			
		<i>Microdochium nivale</i> (Pink snow mold)			
Bio-Save 10LP, 110 ☉	<i>Pseudomonas syringae</i> ESC-10 & ESC-11	<i>Botrytis cinerea</i> , <i>Penicillium</i> spp., <i>Mucor pyriformis</i> , <i>Geotrichum candidum</i>	Pome fruit, citrus, cherries and potatoes	Lyophilized product, frozen cell concentrated pellets added to water to form a liquid suspension and applied postharvest to fruit as drench, drip or spray	EcoScience Corp. (Longwood, FL)
EPA registered 1995					www.villagefarms.com
BlightBan A506 ☉ EPA registered	<i>Pseudomonas fluorescens</i> A506	<i>Erwinia amylovora</i> and russet-inducing bacteria	Almond, apple, apricot, blueberry, cherry, peach, pear, potato, strawberry and tomato	Wettable powder applied as spray at bloom time	NuFarm Inc. (Burr Ridge, IL) www.nufarm.com
Cedemon ☉	<i>Pseudomonas chlororaphis</i> strain	Leaf stripe, net blotch, <i>Fusarium</i> spp., spot blotch, leaf spot	Barley and oats	Seed treatment applied as seed dressing	BioAgri (Uppsala, Sweden)
EPA registered ATeze*	<i>Pseudomonas chlororaphis</i> 63-28	<i>Pythium</i> spp., <i>Rhizoctonia solani</i> , <i>Fusarium oxysporum</i>	Potential for use on cereals Ornamentals and greenhouse grown vegetables	Liquid applied as drench	www.bioagri.se Eco Soil Systems Inc. (San Diego, CA)
EPA registered 2001 FROSTBAN*	<i>Pseudomonas fluorescens</i> A506, 1629RS	Frost-forming bacteria	Fruit crops, almond, potato and tomato	Liquid applied as spray early in growing season	www.ecosoil.com Frost Technology Corporation
EPA registered 1992	<i>P. syringae</i> 742RS				Plant Health Technologies (Lathrop, CA)

Information regarding products highlighted ☉ is adapted from a list compiled by Dr D. Fravel, USDA-ARS 2000, and updated by the APS Biological Control Committee, USA, 2005 (<http://www.oardc.ohio-state.edu/apbscc>).

Information regarding products highlighted \* is adapted from product registration lists compiled by the US Environmental Protection Agency (<http://www.epa.gov>).

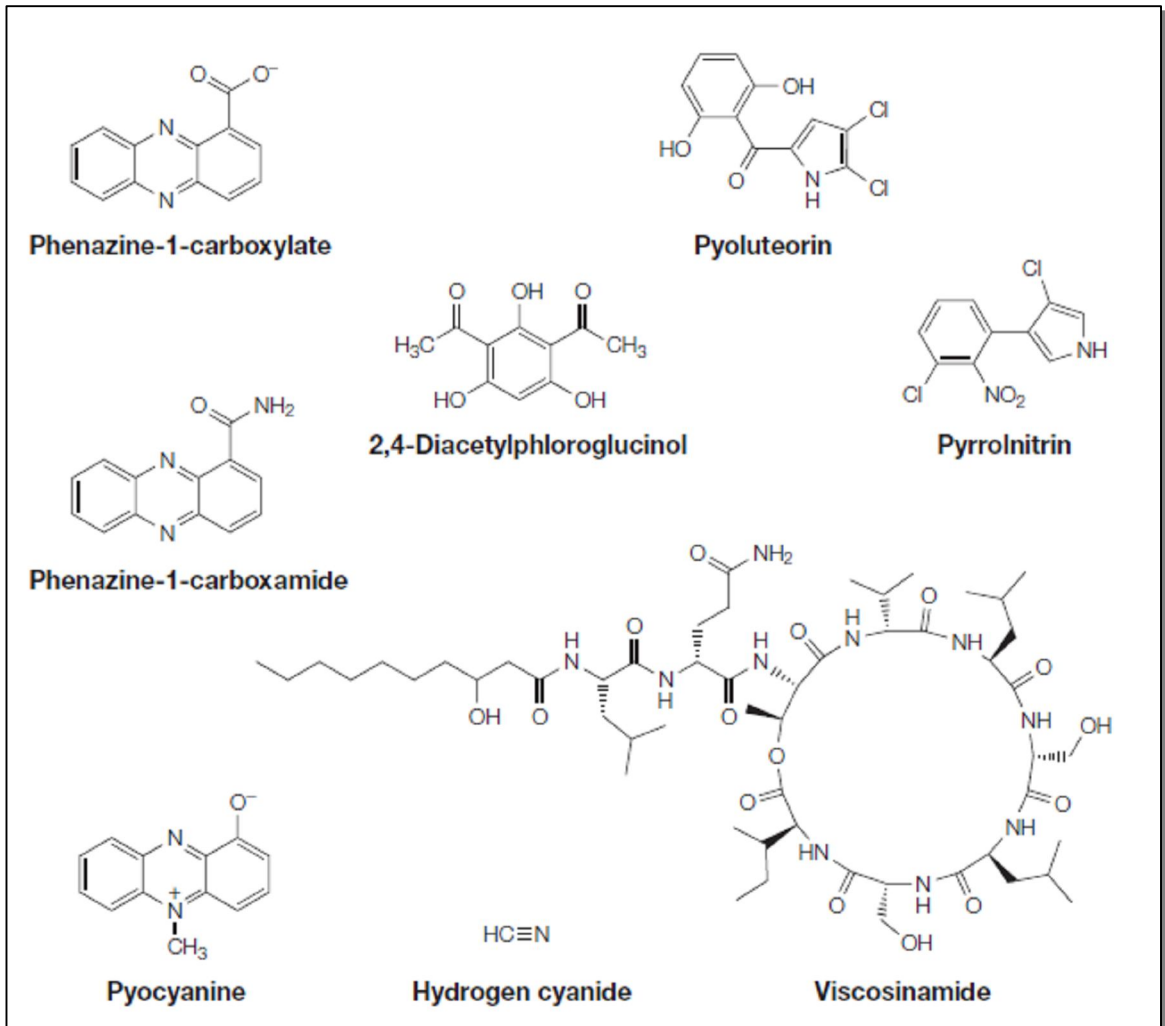


Figure 1.4 Small inhibitory metabolites produced by *Pseudomonas* spp. (Haas and Defago, 2005)

#### 1.4.4 Promotion of plant growth

Bacteria found in the rhizosphere that have positive effects on plant growth are known as Plant Growth-Promoting Rhizobacteria (PGPR). Examples of plant growth-promoting bacteria are *Azospirillum*, *Azotobacter*, *Pseudomonas fluorescens*, and several Gram-positive *Bacillus* (Jaleel et al., 2007). These bacteria can benefit the plants directly by promoting their growth or indirectly through their biocontrol abilities. There is much literature describing the advances of using bacteria with both biocontrol and plant growth promotion activities in the control of plant diseases (Rovira, 1965; Glick, 1995; Welbaum et al., 2004). For instance, promoting plant growth can enhance and speed the seedling growth rate, which in turn reduces the susceptibility time and causes disease escape. The production of phytohormones can impact root structure by overproduction of root hairs and lateral roots, which then increase nutrient and water uptake, thus enhancing plant growth. Most *Pseudomonas* strains possess plant growth-promoting capabilities because of their aggressive root colonization, stimulation of plant growth, and ability to control different plant diseases (Haas and Defago, 2005).

The plant growth stimulation could occur through production of phytohormones (auxin, gibberellins, and cytokinin), nitrogen fixation, phosphate solubilization, and production of volatile stimulants such as ethylene and 2,3-butanediol (Ryu et al., 2003; Vessey, 2003). For example, treatment of Madagascar periwinkle seedlings with *P. fluorescens* increased the biomass yield and alkaloid content. These effects might be due to the phytohormone production (such as gibberelline, and auxin) produced by this *Pseudomonas* strain (Jaleel et al., 2007). In addition, treatment of tomato plants with a strain of *P. fluorescens* known to produce phytohormones has improved plant growth,

reduced root galling resulting from root-knot nematode infection, and affected nematode reproduction (Siddiqui, 2004).

### **1.5 Bacterial cyanide and plant responses.**

Over 2,000 plant species such as cassava, clover, and New Zealand spinach are known to naturally produce cyanide as a defense mechanism against insects and small herbivores (slugs, snails, and voles) that eat these plants (Larsen et al., 2004). These plants produce cyanide as a cyanogenic glycoside. The plants protect themselves from the autotoxicity by storing the cyanogenic glycoside in the vacuoles. When herbivores attack the plant cells, the glycosides are released together with the plant enzymes that convert these glycosides to HCN, which kills the attackers (Rudrappa et al., 2008). Moreover, all vascular plants, fungi, and algae produce cyanide as a co-product during ethylene biosynthesis (Peiser et al., 1984; Piotrowski and Volmer, 2006). During ethylene biosynthesis, one cyanide molecule is produced as a by-product for each ethylene molecule. Plants possess several cyanide-resistant oxidases in their mitochondria to help maintain regular production of ATP and normal plant growth and development (Shugaev, 1999). A majority of vascular plants produce the enzyme, beta-cyanoalanine synthase, which produces beta-cyanoalanine from the HCN and a cysteine molecule. The beta-cyanoalanine is converted by beta-cyanoalanine hydrolase to asparagines, which is known to be involved in nitrogen storage (Castric et al., 1972). To this end, plants that produce these enzymes are capable of detoxifying the harmful HCN and converting it into beneficial molecules. Currently, little information is available about how much cyanide fed from outside the plants can be detoxified using the same pathway.



Microorganisms in the rhizosphere (the soil area around plant roots) significantly influence plant growth and performance by changing soil conditions. Rhizobacteria such as cyanide-producing *Pseudomonas* are good root colonizers, use root exudates for their growth and compete with other soil microorganisms for the available nutrients. These bacteria can influence plant growth positively or negatively depending on plant species, available nutrients, and root exudates (Bakker et al., 1987; Schippers et al., 1990; Zeller et al., 2007). For example, some *Pseudomonas* isolates that have negative effects on bean and pea have no effects on wheat. In addition, the same bacterial isolates show different effects on different pea or bean genotypes (Åström and Gerhardson, 1988). Cyanide is widely produced by several soil-*Pseudomonas* isolates. The level of HCN produced by these isolates at the root level can positively or negatively influence plant growth and development. For instance, *Pseudomonas* isolates that produce high levels of HCN could be considered deleterious bacteria. Although high levels of HCN may protect the plant from being attacked by other plant pathogens, it could lead to inhibition of plant growth. The plant detoxifying machinery may only be able to handle low levels of bacterial HCN. Higher cyanide levels could inhibit several enzymes that are involved in plant metabolic processes. For example, cyanide inhibits enzymes that are involved in respiration (such as cytochrome-c-oxidase), carbon dioxide and nitrate assimilation, carbohydrate metabolism, and defense against reactive oxygen species (Grossmann, 1996)

Several studies have attempted to study the effects of external cyanide on plant growth and development. Smith and Arteca (2000) demonstrated that treating *Arabidopsis* plants with either 50µM HCN for 20min or 9µM for 40min rapidly activated

the 1- aminocyclopropane-1-carboxylic acid synthase gene (ACS6) expression. ACS6 is the first enzyme in the ethylene pathway which produces 1- aminocyclopropane-1-carboxylic acid (ACC) from adenosylmethionine. They suggested that cyanide may be involved in ethylene biosynthesis regulation especially under high HCN concentrations. Rudrappa et al. (2008) showed that HCN produced by different strains of the human pathogen, *P. aeruginosa* (such as PAO1 and PA14), severely inhibited *Arabidopsis* primary root growth. Using *A. thaliana* Col-0 transgenic plants expressing a GUS reporter gene driven by auxin responsive promoter DR5 (DR5::GUS), they demonstrated that bacterial cyanide suppresses the production of the plant hormone, auxin, by interfering with its signaling pathways. Bacterial strains with mutations in the *hcnABC* operon did not affect the root growth.

## 1.6 Dissertation overview

The objective of my dissertation is to provide more insight into the role HCN plays in nematode control. Several nematode-lethal *Pseudomonas* isolates collected and isolated by former members of Dr. Christopher Taylor's laboratory were characterized and their lethality effects on nematodes evaluated. Using these isolates, I will address four important questions: 1) Is HCN production a common trait among all nematode-lethal *Pseudomonas* isolates? 2) Does HCN production operate through the HCN gene cluster? 3) Does HCN from nematode-lethal *Pseudomonas* isolates contribute to the lethality toward free-living and plant-parasitic nematodes? 4) Do nematode-lethal isolates produce HCN in the rhizosphere? The results from this work will address fundamental questions and provide much needed information about whether

*Pseudomonas* are effective biocontrol agents for plant-parasitic nematodes, and whether HCN produced by fluorescent *Pseudomonas* contributes to the biocontrol capabilities.

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## **CHAPTER II**

**Characterization of nematode-lethal isolates of**

***Pseudomonas***

## Contribution of Authorship

All experiments, figures, figure legends, and Materials and Methods selection were conducted or created by Haytham Aly except: the phenazine compound analysis was conducted in Dr. Pat Okubara's (Washington University) laboratory, and isolation and identification of bacterial strains were done by Dr. Joseph Kamalay. The chapter was edited and modified by Dr. Christopher Taylor.

### 2.1 Abstract

**Aim:** To identify and characterize *Pseudomonas* isolates that have broad-spectrum control of plant-parasitic nematodes and other plant pathogens.

**Material and Results:** Using the "model" nematode, *Caenorhabditis elegans* in a high-throughput assay, we have recovered from water, soil or plant samples, 63 different isolates of *Pseudomonas* that display nematode inhibitory activity. About 30% of these *C. elegans*-lethal isolates were also lethal to root-knot nematode (*Meloidogyne incognita*) in an *in vitro* bioassay. In plant/sand experiments, 12 isolates exhibiting activity against root-knot nematode also significantly reduced soybean cyst nematode (*Heterodera glycines*) infestation of soybean plants. All nematode-lethal isolates were characterized on the basis of their motility, exoprotease activity, production of fluorescent metabolites, siderophores, hydrogen cyanide (HCN) and polysaccharides, and presence of genes for the biosynthesis of the antimicrobial metabolites 2,4-diacetylphloroglucinol, phenazine-1-carboxylic acid, pyrrolnitrin and pyoluteorin. Growth inhibiting activity against other plant pathogenic bacteria and fungi were observed for several of the nematode-lethal

strains. Most of the isolates were motile and produced polysaccharides *in vitro*, suggesting their potential to migrate to and associate with the plant root or the nematode. In addition, production of exoprotease, siderophores and HCN was common among the nematode-lethal isolates. Biosynthesis loci for antimicrobial metabolites were detected in 13 (21%) of the 63 isolates, but only five of these also were active against *M. incognita* and three against *H. glycines*.

Conclusion: Numerous strains with broad-spectrum activity against plant-parasitic nematodes were identified. Although nematode-lethal *Pseudomonas* are likely to be motile and producers of siderophores, exoproteases, and HCN, no single biochemical factor accounted for the nematicidal activities of all isolates, suggesting that either multiple factors or an unknown single factor is involved in nematode control.

Significance: This study has identified and characterized several novel and new *Pseudomonas* isolates that have broad spectrum activity against plant pathogenic bacteria, fungi and plant-parasitic nematodes.

## 2.2 Introduction

Plant-parasitic nematodes are among the world's most devastating pests that attack many agronomically important crops, including soybean, corn, wheat and cotton. These nematodes contribute significantly to the loss of crop yield and quality. The estimated value of agricultural damage associated with plant-parasitic nematodes is \$7 to 9 billion annually in the United States (Koenning *et al.* 1999) and over \$77 billion worldwide, which are considered to be conservative estimates by many nematologists

(Sasser and Freckman 1987). Root-knot nematodes (*Meloidogyne* spp.) are obligate sedentary endoparasites that are responsible for approximately 50% of all nematode damage (Sasser, 1980). They are capable of reproducing on over 2,000 different species of plants, including many agricultural crops. The damage to crops caused by various members of the genus *Meloidogyne* has been reviewed (e.g., Sasser and Carter 1985). Symptoms of infection by root-knot nematodes may include knot- or gall-like formations on the roots that inhibit the ability of the root to take up nutrients and water (Dorhout et al., 1991). Damage due to soybean cyst nematodes has risen from \$2.6 billion worldwide (Sasser and Freckman, 1987) to over \$4 billion in the United States alone in 1994 (Wrather, 1995).

Current methods of nematode control entail the use of host genetic resistance, crop rotation and nematicidal chemicals. To date, only a few host resistance genes have been identified that can be used for combating nematodes (e.g., Williamson and Kumar 2006; Lilley et al. 2007). Additionally, the effectiveness of the resistance genes in heterologous plant species is very limited (Williamson and Hussey 1996). Although resistant plant varieties can mount effective defenses against specific nematodes, the resistance is not always durable, and sources for broad-spectrum resistance are not available for many crops (Whitehead 1997). Currently, resistant varieties used in combination with crop rotation are the most widely used method of controlling nematodes. However, a rotation regimen can limit the farmers' choice of crop type or variety. Chemical nematicides can affect many different nematodes, are costly to apply, and as most insecticides, can be harmful to humans, animals and the ecosystem. The broad-spectrum fumigant methyl bromide efficiently controls nematodes and other soil-

borne pests and pathogens, but this chemical is slated to be removed from the market because of its non-specific activity against beneficial soil microorganisms and its potential as a greenhouse gas (Vick 1999; Abawi *et al.* 2000).

Although biological control offers a sustainable means of reducing plant-parasitic nematodes, this approach has met with limited success in the context of modern cropping systems, possibly due to complex interactions between the nematode, its host, other rhizosphere inhabitants and the environment (Barker 2003). Nevertheless, a large number of bacteria have been described in the literature as reducers of nematode populations, including members of the genera *Agrobacterium*, *Alcaligenes*, *Bacillus*, *Clostridium*, *Desulfovibrio*, *Pasteuria*, *Pseudomonas*, *Serratia* and *Streptomyces* (reviewed in Siddiqui and Mahmood 1999). Bacterial biocontrol agents for plant-parasitic nematodes are commercially available (Dong and Zang 2006), and species of the fungus *Trichoderma* have been also examined as a means of suppressing nematode populations (Barker 2003; Siddiqui and Shaukat 2003; Dong and Zang 2006).

The fluorescent pseudomonads are among the best-characterized biocontrol bacteria, are abundant in arable soils and are known to be excellent colonizers of the rhizosphere (Lugtenberg *et al.* 2001). Their activities against plant-pathogenic fungi and to a lesser extent nematodes have been documented (Sulston and Hodgkin 1988; Gallagher and Manoil 2001; Weller *et al.* 2002; Persello-Cartieaux *et al.* 2003; Hass and Défago 2005). Certain strains of *Pseudomonas* produce siderophores that chelate and sequester iron in the rhizosphere, preventing its availability to other organisms (Thomashow 1996; Hass and Défago 2005). *Pseudomonas* also can produce antifungal metabolites (antibiotics) such as 2,4-diacetylphloroglucinol (DAPG), pyrrolnitrin,

pyoluteorin, and the phenazines (reviewed in Hass and Keel 2003; Hass and Défago 2005). The broad-spectrum respiratory inhibitor hydrogen cyanide (HCN) is generated by many strains of *P. fluorescens* and *P. chlororaphis* (Hass and Keel 2003), and HCN is known to be nematicidal (Gallagher and Manoil 2001). Proteases, including an extracellular protease from *P. fluorescens* (Siddiqui *et al.* 2005), are generally considered to exert nematicidal activity by degradation of the cuticle (Siddiqui and Mahmood 1999). Cell surface polysaccharides have been implicated in attachment to host surfaces and in root colonization (De Weger *et al.* 1989; Dekkers *et al.* 1998) and in biofilm formation (Lugtenberg *et al.* 1991) but their roles in interactions with soil-borne nematodes remain uncharacterized. All together, there is limited information about the numbers and types of mechanisms used by *Pseudomonas* spp. for nematode control, and whether these various traits help the *Pseudomonas* persist in a variety of soil types and under biotic and abiotic stress conditions.

We initiated a project to survey members of the genus *Pseudomonas* for nematicidal activity. We sought to investigate common trait(s) or characteristic(s) that might provide insights about the mechanisms underlying nematode control. In this study, we provide a biochemical characterization of 63 *Pseudomonas* isolates that are lethal to *C. elegans*. We show that about a third of these isolates also are lethal to the root-knot nematode *M. incognita* and over half of these reduce infestation of soybean cyst nematode (*H. glycines*) on soybean. The nematode-lethal *Pseudomonas* are likely to be fluorescent and motile, secrete siderophores and exoproteases, and produce hydrogen cyanide. Collectively, these bacterial isolates will be used to address fundamental

questions about the effectiveness of *Pseudomonas* as biocontrol agents of plant-parasitic nematodes.

Information about the characteristics and utility of these nematode-lethal bacteria are valuable, a clear understanding is needed of the diversity of biocontrol mechanisms and how biocontrol bacteria can be rendered more robust and effective under field conditions. In general, there is limited information on the mechanisms that the *Pseudomonas* use to deter or kill nematodes and on the bacterial traits these nematode-lethal isolates require for persistence in a variety of soil types and under biotic and abiotic stress in the field.

### **2.3 Materials and Methods**

#### ***Media***

All bacterial isolates were grown at 30°C in Luria-Bertani (LB) medium (Invitrogen Corp., Carlsbad, CA), King's medium B (KMB) (King *et al.* 1954) or HY-SOY medium unless otherwise indicated. HY-SOY media is the same as KMB except that the peptone component is replaced by soy peptone (Quest International, Hoffman Estates, IL, USA). The *C. elegans* strain used in this study was maintained on nematode growth media (NGM; Lewis and Fleming 1995). The root-knot nematode, *M. incognita*, was maintained on *Arabidopsis* according to Hammes *et al.*, 2005. The soybean cyst nematode, *H. glycines* was maintained on soybean plants in the greenhouse (16hr light:8hr dark; 26 °C) in sand.



### ***Pseudomonas isolates used in this study***

Bacterial isolates were recovered from a variety of locations and source materials for this study. Briefly, three resources were sampled: water from the Missouri and Mississippi rivers containing a high concentration of soil particulates washed from upstream soils; frozen and dried plant specimens from The Missouri Botanical Garden, where over five million plant specimens from around the world are archived; and soils from farms throughout Missouri (kindly provided by Robert Heinz, Extension Nematology Lab, University of Missouri, Columbia, MO, USA), Wisconsin and Wyoming. A total of 500 water, plant and soil samples in our collection were diluted in sterile water. Samples were plated in a dilution series on KMB and HY-SOY media and grown overnight at 30°C. Plates were stored at 4°C for three days to enhance the formation of fluorescent pigments in bacterial colonies. Reference strains of *Pseudomonas* include Pf-5 (kind gift from Dr. Linda Thomashaw) and strains Pfmvp1-4, Q8r1-96, Pf2-79, and PfQ2-87 obtained from the Okubara laboratory. Nematode-lethal isolates can be obtained from the Taylor laboratory under a standard MTA.

### ***Reference bacterial, fungal and nematode strains for inhibition studies***

The *Pseudomonas* strains for the present study are listed in Table 2.1. Strains used for inhibition assays were *Escherichia coli* strain DH5 $\alpha$ , *Bacillus cereus* and *Agrobacterium rhizogenes* strain K599. *E. coli* cultures were grown at 37°C; other bacterial cultures were grown at 30°C. *Fusarium solani*, causal agent of sudden death syndrome in soybean (kind gift from Dr. Dilip Shah, Donald Danforth Plant Science Center, St. Louis, MO, USA), was grown on potato dextrose agar (PDA; Difco; Becton,

Dickson and Company, Franklin Lakes, NJ, USA). The free-living nematode, *C. elegans* strain N2, and the plant-parasitic nematodes, *M. incognita* (root-knot nematode) and *H. glycines* (soybean cyst nematode; HG type 0, Race 3) were also used in this study.

### ***C. elegans in vitro* bioassays**

*C. elegans* bioassays were performed in triplicates in 50  $\mu$ L of bixenic aqueous culture containing the free-living nematode *Caenorhabditis elegans* and an *E. coli* food source. A wild type strain of *C. elegans* was propagated on NGM agar plates inoculated with *E. coli* strain MC1061 [ $F^-$  *araD139 (ara-leu)* 7696 *relA1 spoT1 galE15 galK16 (lac)* *X74 rpsL hsdR2 mcrA mcrB1*] by incubation at 23°C in Parafilm-sealed plastic Petri plates. Egg harvesting and hatching were carried out according to Sulston and Hodgkin, 1988. Individual bacterial colonies from KMB and HY-SOY plates were inoculated into 500  $\mu$ L per well of KMB in 96-deep-well micro-titer plates. Bacterial cultures were grown overnight at 30°C. Five  $\mu$ L of overnight grown bacterial culture was added in triplicate to 20 synchronized L1 stage juveniles of *C. elegans* that were placed in 45  $\mu$ L of S-Full medium (Lewis and Fleming 1995) supplemented with *E. coli* MC1061 cells. The *E. coli* served as a nutrient source to ensure that any inhibition of the nematode growth was not due to starvation or nutritional deficiencies. Five  $\mu$ L of overnight *E. coli* DH5 $\alpha$  culture and dH<sub>2</sub>O were added to 20 L1 stage of *C. elegans* to serve as a negative controls. Nematode populations were evaluated for lethality after incubation at 23°C for up to 7 days. Bacterial isolates exhibiting an LD<sub>100</sub> (lethal dose = 100%) under the described test conditions were restreaked on fresh KMB plates, and single colonies isolated and retested. Isolates were classified as non-lethal (no or limited death after 7 days), slow- (kills all nematodes after 4 days; LD<sub>100</sub>), moderate- (kills all nematodes

between 2- 4 days; LD<sub>100</sub>) or fast- (kills all nematodes on the first day of the assay; LD<sub>100</sub>). Strains Pf-5, Pf mvp 1-4, and Pf Q2-87 (Kraus and Loper 1995) was used as reference strains. All nematode-lethal strains were re-streaked on KMB plates and an individual colony picked and retested. Only those isolates which retested positive for nematode activity were tested further.

### ***Root-knot nematode in vitro bioassays***

*Pseudomonas* isolates lethal to *C. elegans* were tested *in vitro* for activity against the root-knot nematode, *M. incognita*. The root-knot nematode bioassay was identical to that for *C. elegans* except that 40 freshly hatched second-stage juveniles (J2) of axenically-grown *M. incognita* were used for each assay and no *E.coli* MC1061 was added. Isolates were deemed lethal to root-knot nematodes when the nematodes were obviously dead (digested by the bacteria) or became ridged (Fig. 2.1) and failed to move either when prodded with a dental pick or when transferred to fresh NGM plates.

### ***Soybean cyst nematode in planta bioassay***

The *in planta* activity of the root-knot *Pseudomonas* lethal isolates against the soybean cyst nematode, *H. glycines* was performed as described by Siddiqui and Shaukat (2004). Soybean seeds were sterilized by chlorine gas under vacuum desiccator for 18 hrs (2 ml of 37 % (w/w) HCl was added to 100 ml commercial bleach). Sterilized soybean seeds were sown individually in small pots filled with 300 g of unsterilized sand, placed in a growth chamber (26°C; 70% humidity; 600 mol/m<sup>2</sup> light intensity) and sub-irrigated. After 10 days, 15 soybean seedlings were inoculated as a soil drench, with

overnight cultures of *Pseudomonas* isolates resuspended in sterile water at a rate of  $10^4$  cells per gram of sand. Control plants (45 in total) were mock-inoculated using only water. Four days after bacterial inoculation, 4,000 soybean cyst eggs were applied to the sand of each plant. After 4 weeks, shoots were removed and root systems vigorously washed with tap water to dislodge cyst. Root systems were blotted dry and dried in a drying oven ( $55^\circ\text{C}$ ). Root dry-weight was measured for each plant. Nematode cysts were harvested from the sand and counted under a dissecting microscope. The number of cysts per gram of dry root weight was determined. Several strains (14B2, 14B11, 15G2, 48C10) were repeated three times to confirm consistency of the data obtained. The Student's unpaired t-test was performed to determine significance ( $P < 0.05$ ) of cyst nematode number per gram of root when inoculated with different *Pseudomonas* isolates as compared to that of the control (mock-inoculated).

### ***Bacterial and fungal inhibition assays***

Inhibition assays were performed *in vitro* to test the activity of all 63 *Pseudomonas* isolates against the fungal phytopathogen, *F. solani*, and three bacterial strains, *E. coli*, *A. rhizogenes*, and *B. cereus*. For *F. solani* assays, 7-mm diameter agar plugs of fungal culture were transferred to the center of a fresh plate of PDA (Difco) and incubated at room temperature. After three days, 10  $\mu\text{L}$  of an exponentially growing culture adjusted to an  $\text{OD}_{600\text{nm}}$  of 0.2 was spread on one side of the assay plate. The plates were incubated at  $30^\circ\text{C}$  for three days and visually scored for inhibition of fungal growth. For bacterial inhibition assays, 500  $\mu\text{L}$  of overnight cultures of *E. coli*, *B. cereus* or *A. rhizogenes* were used to inoculate 1% LB top agar (held at  $42^\circ\text{C}$ ) and spread onto fresh LB plates. Filter disks (6mm diameter) were dipped into overnight cultures of each

*Pseudomonas* isolate and placed onto the top agar plates. Plates were incubated at 30°C overnight and the zone of bacterial inhibition was measured

### ***Bacterial identification***

Primers that target highly conserved regions of the 16S ribosomal RNA (rRNA) sequences (Shiomi *et al.* 1999) were used to PCR amplify the 16S rRNA from freshly isolated genomic DNA (DNeasy, Qiagen, Valencia, CA, USA). PCR amplified products were sequenced and identified using BLASTN analysis (Altschul *et al.* 1997). The 16S ribosomal rRNA sequence was amplified from control strain Pf-5 (Kraus and Loper 1995).

### ***Motility assay***

Motility assays were performed as described by Mavrodi *et al.* (2006). LB medium with 0.3%, 0.5%, 1.0%, or 1.5% agar were inoculated with 5  $\mu$ L of logarithmically grown cultures of *Pseudomonas* adjusted to OD<sub>600nm</sub> 0.1. Plates were incubated right-side up at 30°C and the diameter of outward expansion of the bacterial colony was measured at 24, 48 and 72 hours to determine motility. Isolates were classified as non-motile (no expansion of the inoculated region), slow ( $\leq$ 30 mm colony diameter) or fast ( $>$ 30 mm colony diameter) within 24 hours on 0.3% agar plates. Strain Pf-5 (Kraus and Loper 1995) was used as reference strain for fast motility.

### ***Polysaccharide production and fluorescence***

Production of polysaccharides and fluorescent compounds was assayed as described by Mavrodi *et al.* (2006). The isolates of *Pseudomonas* spp. were inoculated on Pseudomonas Agar F (Difco; Becton, Dickson and Company, Franklin Lakes, NJ, USA) agar and incubated at 30°C. Polysaccharide production (mucoid phenotype) was scored visually after two days of growth. These plates also were placed on a UV light source and visually scored for fluorescence at two days. Reference *P. fluorescens* strain Pf-5 (Kraus and Loper 1995) was used as a positive control for production of fluorescent compounds.

### ***Exoprotease production***

Bacterial isolates were tested for extracellular protease production according to Sacherer *et al.* (1994). Briefly, cultures were grown overnight in LB at 30°C. Casein proteolyzing activity was tested by inoculating 5 µL of logarithmically-grown *Pseudomonas* cultures adjusted to OD<sub>600nm</sub> 0.1 onto skim milk agar plates (Difco). Plates were incubated overnight at 30°C and zones of clearing around the initial sites of inoculation were measured. *P. fluorescens* strain CHA0 (Voisard *et al.* 1989) was used as a positive control.

### ***Siderophore production***

Siderophore production was determined on chromo azurol S (CAS) plates according to Schwyn and Neilands (1987) with some modifications. Solution I contained 60.5mg CAS dissolved in 50 mL water, Solution II contained 1 mM FeCl<sub>3</sub>·6H<sub>2</sub>O, 10 mM

HCl, and Solution III consisted of 72.9 mg hexadecyltrimethylammonium bromide (HDTMA) dissolved in 40 mL water. Solution I (50 mL) was mixed with 10 mL of Solution II and the resulting mixture slowly added to Solution III. The resulting dark blue reagent was autoclaved at 121°C for 15 minutes, mixed carefully to avoid air bubbles with 900 mL of KMB agar at 50°C (CAS) and poured into Petri plates. The CAS plates were inoculated with the *Pseudomonas* isolates and incubated at 30°C for two days. Siderophore production was determined by observing the orange halos around bacterial colonies. Isolates were classified as non-producers (no orange halos) or producers (weak to strong orange halos) after 48 hours on CAS agar.

### ***HCN production assays***

HCN production was measured both qualitatively and quantitatively. The qualitative assay was performed according to Feigel and Anger (1966). Briefly, five mg of copper ethylacetoacetate and 5 mg of 4,4'-methylenebis (*N,N*-dimethylaniline) were dissolved in 2 mL of chloroform to form the HCN detection solution (colorless). Filter disks (6mm diameter) were moistened with a drop of HCN detection solution and placed on the inner lids of LB medium plates inoculated with the *Pseudomonas* isolates. The plates were incubated overnight at 30°C. The appearance of a blue color on the detection paper indicated HCN production.

Production of HCN was quantitatively assayed using a micro-cyanide-ion selective electrode (Lazar Research Laboratories, Los Angeles, CA, USA) as described by Zlosnik and Williams (2004) with some modifications. Fifteen mL of LB medium

were placed into 50 mL conical tubes and inoculated with each *Pseudomonas* isolate. Four mL of 4 M NaOH was added to 15 mL conical tubes and placed within the 50 mL tubes to trap the HCN. Cultures were incubated for two days at 30°C. A 0.1M stock solution of potassium cyanide (KCN, Fluka Chemika/Biochemika, Buchs, Switzerland) dissolved in 4 M NaOH was made immediately before use. The stock solution was serially diluted to  $10^{-2}$  M,  $10^{-3}$  M,  $10^{-4}$  M and  $10^{-5}$  M KCN for a standard curve. HCN content of the 4 M NaOH trap solution from each *Pseudomonas* culture was quantified using the cyanide electrode and the KCN standard curve. HCN measurements were taken on three biological replicates of each isolate.

#### ***Detection of secondary metabolite biosynthetic genes using PCR***

*Pseudomonas* isolates were cultured for 16 to 18 hours in 3 mL of LB medium at 28°C, 200 rpm. Cultures (2.5  $\mu$ L) were used directly in PCR reactions containing 0.2 mM of each deoxynucleotide, 2 mM MgCl<sub>2</sub>, 2 pmol of each primer, and 0.2 U Flexi-Taq (Invitrogen Corp., Carlsbad, CA) in a total volume of 25  $\mu$ L. PCR was performed using a PTC-200 thermocycler (MJ Research, Inc., Waltham, MA). The amplification protocol for *phlD* and *phzCD* was one cycle of 95°C for 3 minutes followed by 35 cycles of 94°C for 1 minute, 60°C for 1 minute, 72°C for 1 minute. The amplification of *pltB* and *prnC* was the same, except that the initial denaturation was performed at 94°C for 2 minutes, and annealing was done at 58°C for 45 seconds. PCR primers B2BF 5'-ACCCACCGCAGCATCGTTTATGAGC-3' and BPR4 5'-CCGCCGGTATGGAAGATGAAAAAGTC-3' were used to amplify *phlD* (McSpadden



Gardener *et al.* 2001); PCA2a 5'-TTGCCAAGCCTCGCTCCAAC-3' and PCA3b 5'-CCGCGTTGTTTCCTCGTTCAT-3' for *phzCD* (Raaijmakers *et al.* 1999); PltBf 5'-CGGAGCATGGACCCCCAGC-3' and PltBr 5'-CGGAGCATGGACCCCCAGC-3' for *pltB* (Mavrodi *et al.* 2001); and PrnCf 5'-CCACAAGCCCGGCCAGGAGC-3' and PrnCr 5'-GAGAAGAGCGGGTCCATGAAGCC-3' for *prnC* (Mavrodi *et al.* 2001). *P. fluorescens* strains Q8r1-96 (Raaijmakers *et al.* 1999; Mavrodi *et al.* 2006), 2-79 (Weller and Cook 1983) and Pf-5 (Kraus and Loper 1995) served as positive controls for DAPG, PCA, and pyrrolnitrin and pyoluteorin biosynthetic loci, respectively. PCR products were partitioned on 1.5 % agarose gels. Expected product sizes were 629 bp (*phlD*), 1150 bp (*phzCD*), 773 bp (*pltB*) and 719 bp (*prnC*).

### ***Correlation analysis***

Pair-wise correlation was performed using Pearson's correlation coefficient analysis (SAS vers. 9.3, SAS Institute, Inc., Cary, NC). The number of isolates harboring one trait was used to calculate the expected number of isolates harboring a second trait, assuming that the second trait was independent of the first (null hypothesis).

## 2.4 Results

### *Identification of Pseudomonas isolates lethal to C. elegans*

Bacterial isolates recovered from 500 herbarium, water and soil samples were cultured and their activity against *C. elegans* determined. On average, 16 individual isolated bacterial colonies (single colonies with different color, morphologies, or altered sized colonies) were tested per sample. In total, 768 bacterial isolates were isolated from water, 1,440 from frozen herbarium samples, 2112 from dried herbarium samples and 3,660 from soil samples and tested in the high-throughput *C. elegans* assay (Fig. 2.2). Isolates were considered lethal to *C. elegans* if all nematodes were killed (LD<sub>100</sub>) during the allotted bioassay time (7 days) in each of the triplicated wells. Of the nearly 8,000 bacterial isolates screened, 350 displayed inhibition of *C. elegans* development *in vitro*. BLASTN analysis of 16S ribosomal DNA segments from the *C. elegans* lethal isolates (data not shown) indicated that these isolates could loosely be grouped into three major classes: 73% (249 isolates) are *Bacillus* spp., 18% (63 isolates) are *Pseudomonas* spp., and 9% (30 isolates) represent other bacterial species. The 63 isolates identified as *Pseudomonas* were further examined in this study.

*Pseudomonas* isolates were further characterized for their killing characteristics (Table 2.1). Thirty isolates (48%) killed *C. elegans* in the first day of the assay. Eleven isolates (18%) were considered moderate killers while 22 of the isolates were slow killers (34%) taking up to seven days to kill all nematodes within the well. The end fate for all *C. elegans* was the complete digestion/dissolution of nematode bodies by the overgrowth

of bacteria in the well. The biocontrol strains Pf-5, Pf.mvp 1-4 and Pf Q2-87 were all fast killers of *C. elegans*.

### ***In vitro lethality to root-knot nematodes***

To determine whether the *C. elegans*-lethal *Pseudomonas* isolates were also lethal to root-knot nematode, we challenged second-stage juveniles (J2) of *M. incognita* with whole bacteria and examined juveniles for movement at five days post-incubation. J2 nematodes mock-inoculated with water or *E. coli* were active and moving at the end of the assay period (Fig. 2.1A). Of the 63 *C. elegans*-lethal strains, 20 (30%) were lethal to *M. incognita* juveniles (Figure 2.3, Table 2.1). Biocontrol strains Pf-5, Pf.mvp 1-4 and Pf Q2-87 were not lethal to root-knot nematode. Post-mortem analysis showed both nonreversible paralysis and complete rigidity of the nematode body or complete digestion and dissolution of the nematode body. We found that all nematodes that exhibited rigidity at day five of the bioassay failed to have their motility restored when removed away from the bacteria, transferred to fresh NGM plates or poked with a sharp dental pick.

### ***In vivo lethality to soybean cyst nematodes***

Due to time and space limitations only those 20 *Pseudomonas* isolates that showed *in vitro* activity against *C. elegans* and *M. incognita* were tested in a whole plant assay against the soybean cyst nematode, *H. glycines*. Twelve (60%) of the twenty isolates ( 14B2, 15D11, 15G2, 28B5, 39A2, 48C10, 48D1, 88A6, 88B11, 90F12, 91D1, and 528C10) significantly ( $P < 0.05$ ) reduced the final population densities of cysts per plant as compared to the control (Fig. 2.4). Shoot growth was uniform among all treatments and controls. However, several isolates had increased root weight (data not

shown) as compared to the controls suggesting that these isolates may have root-promoting properties.

### ***Fungal and bacterial growth inhibition assays***

Potential antibacterial activity of each nematicidal *Pseudomonas* isolate was tested using three different bacteria, *E. coli* (a Gram-negative enteric bacterium), *B. cereus* (a Gram-positive food-borne pathogen) and *A. rhizogenes* (a Gram-negative soil-borne plant pathogen). Anti-bacterial activity was based on a zone of growth inhibition (clear ring) around the *Pseudomonas* colony. Of the 63 isolates tested, 11 (17%) showed activity against *E. coli*, 26 (41%) against *A. rhizogenes* and 34 (54%) against *B. cereus* (Figure 2.3; Table 2.1). Furthermore, twenty isolates inhibited the growth of more than one bacterium and eight inhibited the growth of all three bacteria tested.

Activity of the *Pseudomonas* isolates against the plant-pathogenic fungus *F. solani* was evaluated based on an observable reduction of growth of fungal hyphae toward the *Pseudomonas* colony. In our study, 25 isolates (39%) exhibited antifungal activity (Figure 2.3; Table 2.1). Interestingly, the growth of three isolates, 528C10, 48B7, and 91D1, was inhibited by *F. solani*. Four isolates; 3D6, 12H11, 15H10 and 36C6, showed activity against *F. solani* and all three bacteria. The biocontrol strain Pf-5 showed activity against all bacteria and fungi tested as expected though did not exhibit any significant activity against plant-parasitic nematodes.

### ***Motility***

We tested the ability of 63 *C. elegans*-lethal isolates of *Pseudomonas* to migrate on semi-solid LB medium. Motility was scored according to the diameter of the bacterial colony after 24 hours of growth on 0.3% LB agar. These conditions were selected

because differences among the isolates were obvious by 24 hr and at agar concentrations greater than 0.3%, most isolates were unable to migrate from the initial colony. We observed that 49 of the isolates (78%) were motile on the semi-solid LB agar. Twenty nine isolates and the Pf-5 control exhibited fast motility (>30mm of outward growth in 24 hours), 21 exhibited slow motility (<30mm) and 13 were non-motile (Table 2.1). All herbarium isolates were classified as highly motile, whereas 63% and 25% of the isolates collected from water and soil samples, respectively, were placed in this class.

### ***Polysaccharide, exoprotease and siderophore production***

To test for the production of polysaccharides, we examined the 63 nematocidal *Pseudomonas* isolates for their ability to produce mucoid colonies on *Pseudomonas* Agar F (Difco). After 48 hours of growth, 36 of the 63 isolates (56%) exhibited a mucoid phenotype (Fig. 2.5; Table 2.1). Forty seven (75%) of the herbarium isolates were polysaccharide producers. Polysaccharide production was observed in 23 and 40 (37% and 64%) of isolates from water and soil samples, respectively.

Fifty of the 63 (79%) isolates exhibited exoprotease activity based on their ability to proteolyze milk casein (Fig. 2.5; Table 2.1). One-half (50%) of the herbarium isolates were exoprotease producers, whereas 89% and 80% of the isolates from water and soil samples demonstrated exoprotease activity.

Siderophore production in our *Pseudomonas* isolates was assessed by conversion of the blue pigment in CAS medium to orange after 48 hr of growth. Of the 63 isolates, 49 (77%) produced siderophores (Fig. 2.5; Table 2.1). Fourteen isolates were considered non-producers (no orange halos; class 0), 11 were weak producers (small orange halos;

class 1), 17 were moderate producers (bright distinctive orange halos; class 2), and 21 were strong producers (plate was entirely orange; class 3) (Table 2.1). Siderophore production was prevalent in isolates from water and soil samples, at 84% and 77%, respectively, whereas 62% of the herbarium isolates were siderophore producers.

### ***Fluorescent pigment production***

Fluorescent compounds were observed in 49 of 63 (78%) *Pseudomonas* isolates after culture on *Pseudomonas* Agar F plates. These compounds rendered the colonies orange, orange-yellow, blue, green, blue-green or violet blue under UV light (Fig. 2.5; Table 2.1). Certain isolates produced soluble fluorescent pigments that diffused into the media including the control strain Pf-5.

### ***Detection of antibiotic biosynthesis genes***

PCR-based assays were conducted to detect genes encoding biosynthesis enzymes for the antimicrobial metabolites DAPG, PCA, pyrrolnitrin and pyoluteorin. Thirteen of the 63 isolates harbored one or more gene (Table 2.1). DAPG and pyrrolnitrin genes were most prevalent, occurring in 8 and 7 of the 63 isolates, respectively, whereas the PCA gene was detected in 3 isolates and the pyoluteorin gene in 2 isolates (Table 2.1). Isolate 1B1 was positive for three of the four genes, and isolates 1F2, 14B11, 48B8 and 48G9 each carried two genes. The DAPG and PCA genes were mutually exclusive in our isolate collection.

### ***HCN production***

Production of HCN by 63 isolates nematocidal to *C. elegans* was qualitatively evaluated on the basis of the appearance of blue color on HCN detection paper. Of the 63 *Pseudomonas* isolates screened, 39 (62%) produced HCN (Fig. 2.5; Table 2.1). HCN production was observed in 79% and 72% of the isolates from water and soil samples, respectively, whereas 25% of herbarium isolates produced HCN.

A quantitative HCN assay was conducted using a micro-cyanide probe to authenticate the qualitative assay and to measure the maximum level of the HCN produced by each isolate after two days. The HCN standard curve ( $r=0.99$ ) was used for the quantification. Total HCN produced by each isolate was reproducible among triplicate samples (Table 2.1). An arbitrary cut-off for categorizing an isolate as a HCN producer was set at 1  $\mu\text{mol}$ , the amount determined to be lethal to *C. elegans* in a study by Gallagher and Manoil (2001). HCN production varied among the isolates, ranging from 1  $\mu\text{mol}$  to 731  $\mu\text{mol}$ s (Table 2.1). Fifty-five isolates (87%) were classified as HCN producers using the quantitative assay. Fifteen isolates identified as HCN producers (many of them strong) from the qualitative assay were classified as non-HCN producers using the HCN detection paper.

### ***Correlative analysis of measured Pseudomonas traits***

We sought to determine if any of the measured traits were correlated (either positively or negatively) using Pearson's correlation coefficient analysis (SAS Institute, Inc.), which assigned probabilities that an isolate displaying one trait randomly harbored a second trait; the null hypothesis was that the two traits were unlinked. Only those traits

tested for all 63 isolates were considered for this analysis (lethality to soybean cyst nematode was not considered since only 20 isolates were tested). The p-values for each pair-wise analysis are shown in Figure 2.6. Control of *F. solani* was significantly ( $p=0.047$ ) correlated to HCN production. A correlation was noted between inhibition of *A. rhizogenes* and *E. coli* ( $p=0.002$ ) in the collection of *Pseudomonas* isolates. Inhibition of *A. rhizogenes* and *E. coli* also were significantly correlated with siderophore production at  $p=0.000$  and  $p=0.003$ , respectively. Siderophore production and root-knot nematode inhibition were correlated ( $p=0.041$ ), but inhibition cannot be attributed to iron sequestration under our Fe-rich conditions used for the assays. Many *Pseudomonas* isolates produce fluorescent compounds, exoprotease and HCN, so the significant correlations between exoprotease and HCN ( $p=0.011$ ) and fluorescence and HCN ( $p=0.024$ ) are not unexpected. Though common to most of the *Pseudomonas* isolates tested, exoprotease, siderophore, and polysaccharide production were not correlated with each other. Inhibition of bacterial growth and root-knot nematodes also was uncorrelated. Furthermore, lethality to root-knot nematodes was not associated with motility or production of polysaccharides, antimicrobial metabolites, exoprotease or HCN.



## 2.5 Discussion

We recovered *Pseudomonas* isolates from 500 different plants, water and soil samples and identified 63 isolates that were lethal to the model nematode *C. elegans* in our high-throughput bioassay. Twenty of the 63 isolates were also lethal to the plant-parasitic root-knot nematode *M. incognita*, and 13 of these significantly reduced *H. glycines* infestation on soybean. Additionally, several inhibited the growth of other plant pathogens, bacteria and/or fungi, suggesting that certain isolates exert a broad spectrum of action. This broad-spectrum inhibition, combined with possible plant colonization potential, makes these isolates desirable in future biocontrol studies.

One question we wished to address in this study is whether the “model” nematode, *C. elegans*, is a useful tool for identifying bacteria that have activity against plant-parasitic nematodes. The feeding behavior of the *C. elegans* is different from that of the *M. incognita*. The *C. elegans* have been extensively used as a model to study bacterial pathogenesis in high-throughput experimentation (reviewed in Kaletta and Hengartner 2006). The lifestyle of *C. elegans* differs from that of the plant-parasitic nematodes. The *C. elegans* can feed on and ingest the bacteria as their sole source of nutrients, whereas the plant-parasitic nematodes are obligate parasites, requiring a host for their survival making the high-throughput analyses difficult. Thus, we chose *C. elegans* as a model for our *in vitro* assays. Of the 63 *Pseudomonas* isolates identified, nearly 20% showed *in planta* activity against a plant-parasitic nematode. The rate of lethal bacterial isolate identification from our high-throughput *C. elegans* bioassay was <4.3 %. Due to the obligate nature of plant-parasitic nematodes, bacteria have different ways that cause the lethality. In addition, some bacterial isolates took longer than others

to kill the *C. elegans*, suggesting that build up of the lethal factor is necessary prior to the death of the nematodes. Unlike *C. elegans*, *M. incognita* is a plant parasite that feeds only on plant tissues. Our results showed that all the bacterial isolates that were lethal to *M. incognita* produce HCN. Because of the inability to screen large number of bacterial isolates *in planta*, the use of *C. elegans* as a prescreen for identifying nematode-lethal isolates increases the likelihood of identifying bacteria lethal to plant-parasitic nematodes.

As part of our analysis we wanted to determine the best resources (i.e. plants, soil, or water samples) for identifying nematode-lethal bacteria. On average our “hit” rate for identifying nematode-lethal isolates was <4.3%, of which the bulk of the nematode-lethal bacteria isolated were related to *Bacillus* (Fig. 2.2). This is not surprising because most of the material tested came from dried herbarium collected plant samples and soil, both resources favoring bacteria that can persist during periods of drying. It is interesting to note that one can increase the likelihood of identifying a bacterial isolate that is lethal to nematodes by testing dried materials. Bacteria isolated from samples stored in the herbarium collection at the Missouri Botanical Garden produced a preponderance of *Bacillus* related bacteria. Presumably these bacteria develop from spores that can persist for long periods of time and are present on the collected plant material. Numerous strains of Gram-positive bacteria related to *Pasteuria* and *Bacillus* have been characterized for their lethal effects on nematodes. Some of these have been further characterized for their potential for plant-parasitic nematode control (reviewed by Tian *et al.* 2007)

Since *Pseudomonas* are susceptible to drying (Morrison and Hammer 1941) moist samples (i.e. water, frozen herbarium plant samples, and soils) would increase the

likelihood of identifying *Pseudomonas* that are lethal to nematodes. Overall the best resource for identifying *Pseudomonas* nematode-lethal isolates was from water samples. Moreover, most of the *Pseudomonas* identified from water samples were fast-killers of nematodes and tended to be highly motile. It is interesting to speculate whether the nematode-lethal isolates of *Pseudomonas* found in water samples have adapted to water life by being able to quickly move to a potential host nematode, attack, and kill.

*Pseudomonas* are members of root-associated bacteria, collectively called rhizobacteria, that have evolved mechanisms for infecting, killing and suppressing other rhizosphere inhabitants. Members of the genus *Pseudomonas* have been extensively investigated in the context of control of fungal and bacterial plant pathogens (reviewed in Walsh *et al.* 2001). Biological control is attributed to the ability of *Pseudomonas* spp. to stimulate plant growth, aggressively colonize the host rhizosphere, and directly or indirectly protect plants against soilborne pathogens. The potential of rhizobacteria to control nematode pests, such as soybean cyst and root-knot nematodes (Kloepper *et al.* 1992), has received much consideration in the past few decades. Among the nematicidal rhizobacteria, a majority is represented by species of *Pseudomonas*, *Bacillus*, and *Streptomyces* (Emmert and Handelsman 1999; Pusey 1999; Weller *et al.* 2002). For example, *P. chlororaphis* has been reported to suppress the root-lesion nematode *Pratylenchus penetrans* (Hass and Défago 2005), and *P. aeruginosa* and *P. fluorescens* strain CHA0 reduced gall formation on roots grown in soils infested with root-knot nematodes (Hanna *et al.* 1999; Ali *et al.* 2002; Siddiqui and Shaukat 2003; Siddiqui *et al.* 2005; review by Dong and Zang 2006). Nematicidal biocontrol organisms are commercially available (Dong and Zang 2006).

Broad spectrum biocontrol activities (approximately 74% of *Pseudomonas* isolates) were observed for many of our *Pseudomonas* isolates. Isolate 37A11 has activity against *M. incognita*, *B. cereus*, *A. rhizogenes* and *E. coli*. Isolates 14B2 and 48D1 exhibited activity against all plant pathogens tested, *M. incognita*, *H. glycines*, *F. solani* and *A. rhizogenes*. *Pseudomonas* isolates 91D1 and 88A6 gave the strongest level of protection (88 and 78% reduction, respectively; Fig. 2.4) against soybean cyst nematode and both were from different Missouri soil samples. Microorganisms in suppressive soils have been partially characterized and *Pseudomonas* has been identified within these samples; however it is unknown how much they contribute to their suppressive effects (Borneman and Ole Backer 2007).

As part of our analysis we collectively examined the 63 nematode-lethal *Pseudomonas* isolates for traits linked with biocontrol. Rhizobacteria have evolved numerous mechanisms for infecting and killing nematodes (Hanna *et al.* 1999; Siddiqui and Mahmood 1999; Kerry 2000). Additional traits proposed to be involved in nematode-lethality include exoprotease production (Siddiqui *et al.* 2005), HCN production (Hass and Défago 2005) and small metabolite production (Hamdan *et al.* 1991; Freedman *et al.* 1989; Henry *et al.* 1991). Other traits associated with pathogenicity including motility, polysaccharide, and siderophore production were also examined.

Our results show that bacterial isolates that were lethal to *C. elegans* have different rates of lethality. Strains associated with water appear to kill the nematode very quickly while those associated with soil take longer for lethality to manifest. We speculate that these differences suggest either different mechanisms for killing

nematodes, or alternatively, that slow-killing isolates took longer to build up lethal factor(s) necessary for death. Larger more comprehensive mutagenesis experiments will be needed to determine whether a common killing mechanism exists or multiple mechanisms are available in the *Pseudomonas* genera.

Fifty five (87%) of the *C. elegans*-lethal *Pseudomonas* isolates from water and soil samples produced HCN. The prevalence of HCN production has been reported for other collections of fluorescent pseudomonads (e.g., Kremer and Souissi 2001; Rezzonico *et al.* 2007 27, 44). The levels of HCN production varied among our isolates and were influenced by the oxygen level and the growth medium. Some differences were noticed between the qualitative and quantitative assays that cause false positive or negative results. The reasons for that may be due to culture conditions (liquid media for quantitative and agar media for qualitative measurements), endogenous levels of HCN production below the threshold of detection of the filter paper assays, and exogenous airborne compounds that might cause false positive or negative results. The quantitative measurements, done in triplicate using a sensitive and specific cyanide detector, were highly reproducible among replicated data sets. The broad-spectrum action of HCN on the respiratory electron transport chain may account for the nematicidal activity of our *Pseudomonas* isolates, but HCN was clearly not a common metabolite among all the isolates. HCN production may be detrimental to the plant and could explain the finding that only 25% of the isolates cultured from plant samples were HCN producers. Our results showed that all bacterial isolates lethal to *M. incognita* produce HCN albeit at different levels. However, HCN cannot be the only factor for killing nematodes since

numerous other isolates which are not lethal to root-knot and soybean cyst nematode also produce HCN.

Approximately 74% of the root-knot nematode lethal isolates were exoprotease producers. This too cannot be the only factor for killing the nematodes, since numerous isolates not lethal to *M. incognita* or *H. glycines* are exoprotease producers. However, we cannot necessarily rule out exoproteases given they may have different specificities and modes of action

*Pseudomonas* spp. that produce HCN in combination with other secondary metabolites and/or enzymatic proteins are not uncommon (Kraus and Loper 1995; Mavrodi *et al.* 2001; Ramette *et al.* 2006; Rezzonico *et al.* 2007). In our collection, we noted that six of 13 isolates harbored two or three biosynthetic genes for DAPG, PCA, pyrrolnitrin or pyoluteorin. Exoprotease and HCN have both been identified as potential markers for lethality by other laboratories (Laville *et al.* 1998; Siddiqui *et al.* 2005), but the role of this combination in combating nematodes remains to be determined. DAPG and PCA loci were mutually exclusive among the isolates.

The prevalence of fluorescent pigment production among our isolates is not surprising given that the diffusible fluorescent siderophore pyoverdinin is produced by a majority of fluorescent pseudomonads (Hass and Défago 2005). It has been suggested that phenazines may help mobilize iron from the rhizosphere (Hass and Défago 2005). The *phz* biosynthetic locus was detected only in a small portion of our nematicidal isolates. Interestingly, siderophore production was also correlated with antibiotic activity against *A. rhizogenes*. This indicates the possibility that iron is especially critical for the growth of *A. rhizogenes*.

The ability of the biocontrol agent to inhibit pathogenic microorganisms and to survive in the soil can be attributed to its ability to compete with other organisms for nutrients such as iron. Nearly all living organisms require iron for redox reactions, protein structure, and enzymatic and organellar function (Huertas *et al.* 2006). Results from our *in vitro* experiments showed that the majority of the *C. elegans*-lethal *Pseudomonas* isolates produce siderophores, compounds that sequester iron from the surrounding environment. Interestingly, siderophore production was correlated with root-knot nematode lethality, however given the iron rich conditions of the assay the lethality is not likely due to a loss of usable iron. The possibility that iron (or iron siderophores) play a role in pathways that control nematode lethality can not be ruled out. The nematicidal activity of siderophore-producing isolates will need to be measured under iron-limiting conditions.

Attachment and colonization of biocontrol organisms to plant roots is a limiting step in biological control of pathogenic fungi (Schippers *et al.* 1987; Raaijmakers *et al.* 1999), and is influenced by chemotaxis, motility, and lipopolysaccharide production (Turnbull *et al.* 2001; de Weert *et al.* 2002). Motility and polysaccharide production are common among the fluorescent pseudomonads, and in this study were common among our *C. elegans*-lethal isolates. Motility and polysaccharide production were especially prevalent in isolates collected from plant (herbarium) samples. Motility may be an important fitness trait for activity against migratory stages of nematodes. Likewise it has been shown in other *C. elegans* pathogen systems that biofilm formation, of which polysaccharides are an important component, governs the pathology of the nematode

(Darby *et al.* 2007). However, as indicated by our correlation analysis, motility and polysaccharides were not correlated with activity against root-knot nematode.

Correlative analysis indicates that although motility and exoprotease, siderophore, HCN, fluorescent compound and polysaccharides production are common among the *C. elegans*-lethal isolates, they were not sole predictors for lethality to nematodes. This suggests that an appropriate factor for lethality to plant-parasitic nematodes has not been identified or, alternatively, members of the *Pseudomonas* genus have more than one way to kill nematodes. We favor the latter hypothesis because exoproteases and HCN have both been identified as potential factors for lethality by other laboratories (Laville *et al.* 1998; Siddiqui *et al.* 2005). The biochemical characteristics examined in this study are a subset of known antimicrobial factors. For instance, cyclic lipopeptide surfactants (de Souza *et al.* 2003) are of special interest as nematicidal agents based on their potential to breach the nematode cuticle.

Collectively, our 63 bacterial isolates can be used to address some fundamental questions about the *Pseudomonas*-nematode interaction. Our findings provide a first step to identifying nematicidal mechanisms exerted by *Pseudomonas* spp. and pose additional questions: How well do our isolates colonize roots of plants? How does colonization change upon nematode infection? At what stage of nematode development are the bacteria exerting their lethality? Future work is needed to provide more information about which isolates are most effective against a broader spectrum of plant-parasitic nematodes, whether these share common biochemical characteristics, and if any shared characteristics can be used to select and develop successful biocontrol measures.



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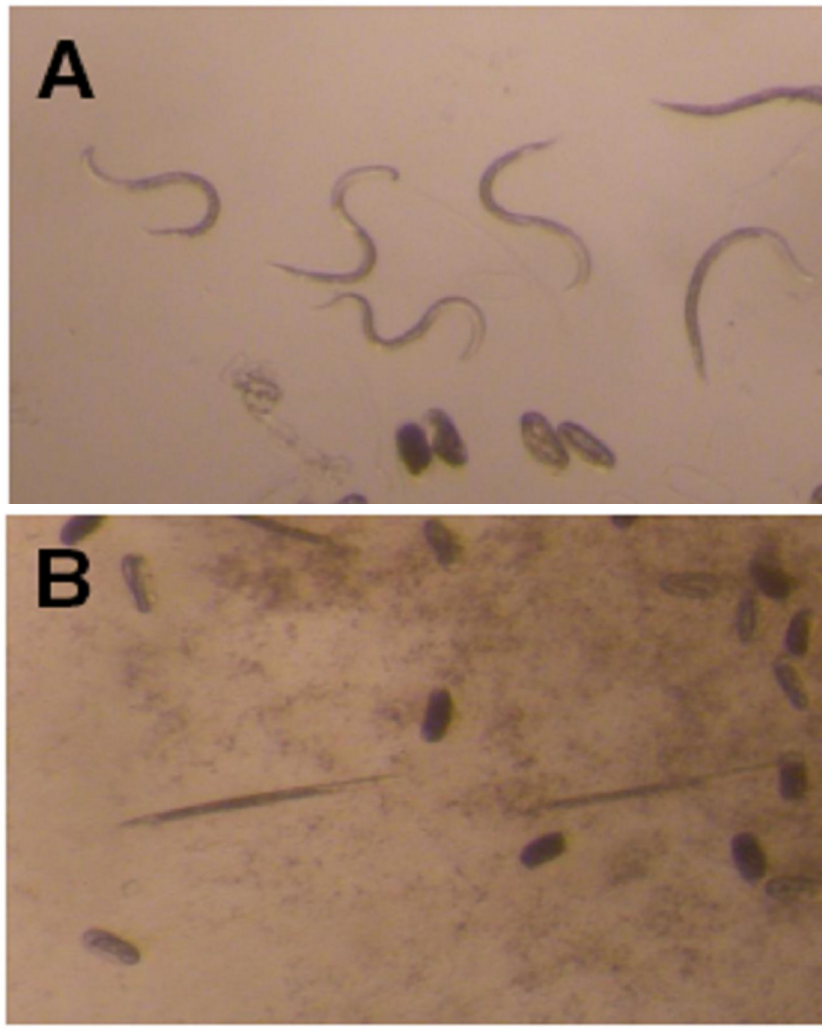
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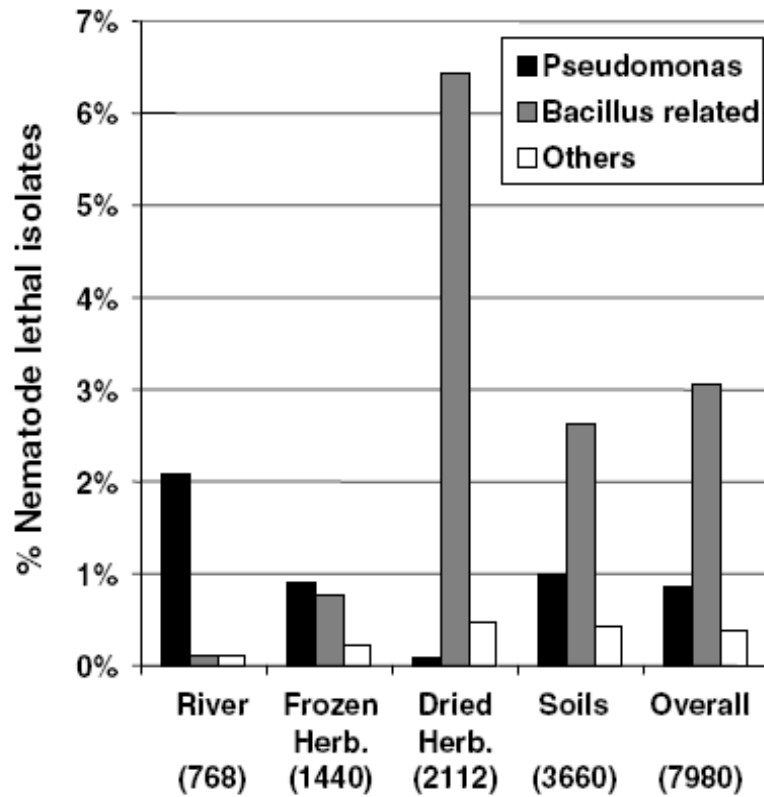
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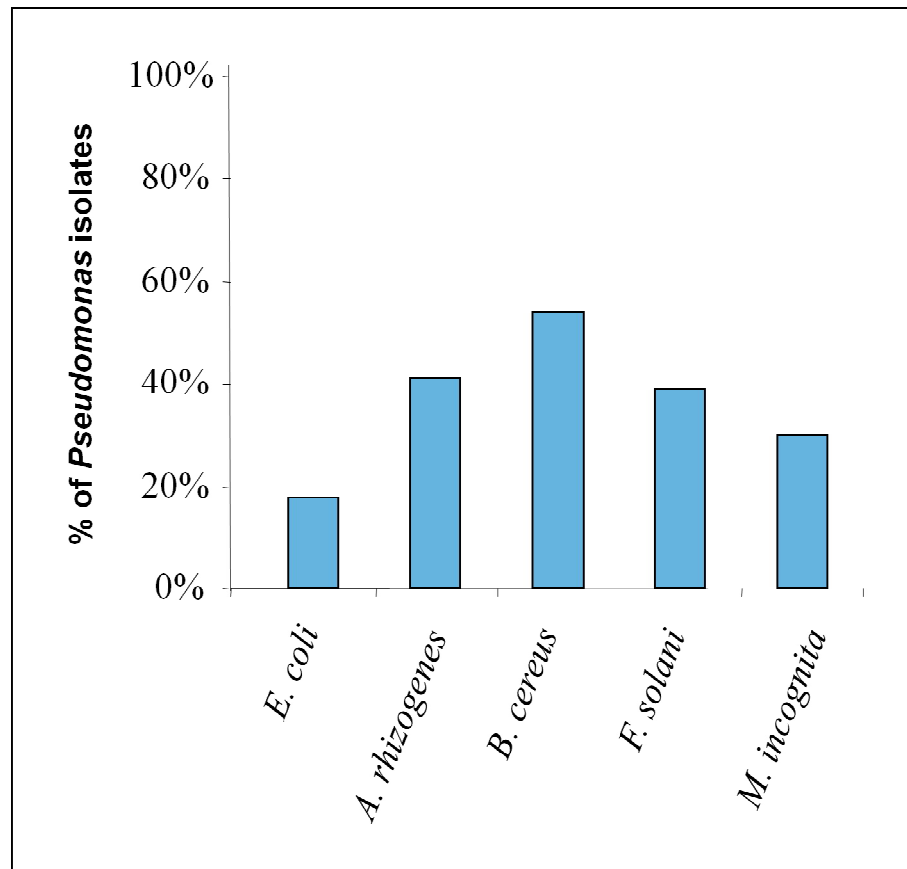
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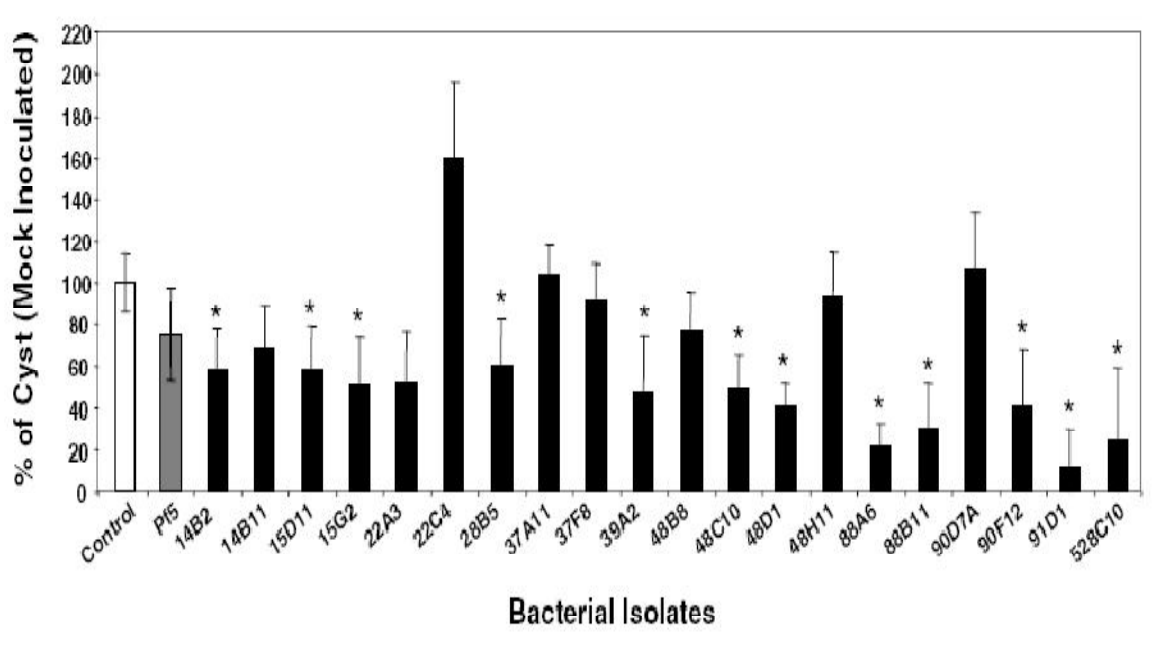
**Figure 2.1** Root-knot nematode lethality assay. **A)** Example of a root-knot nematode non-lethal isolate. **B)** Example of a root-knot nematode lethal isolate.



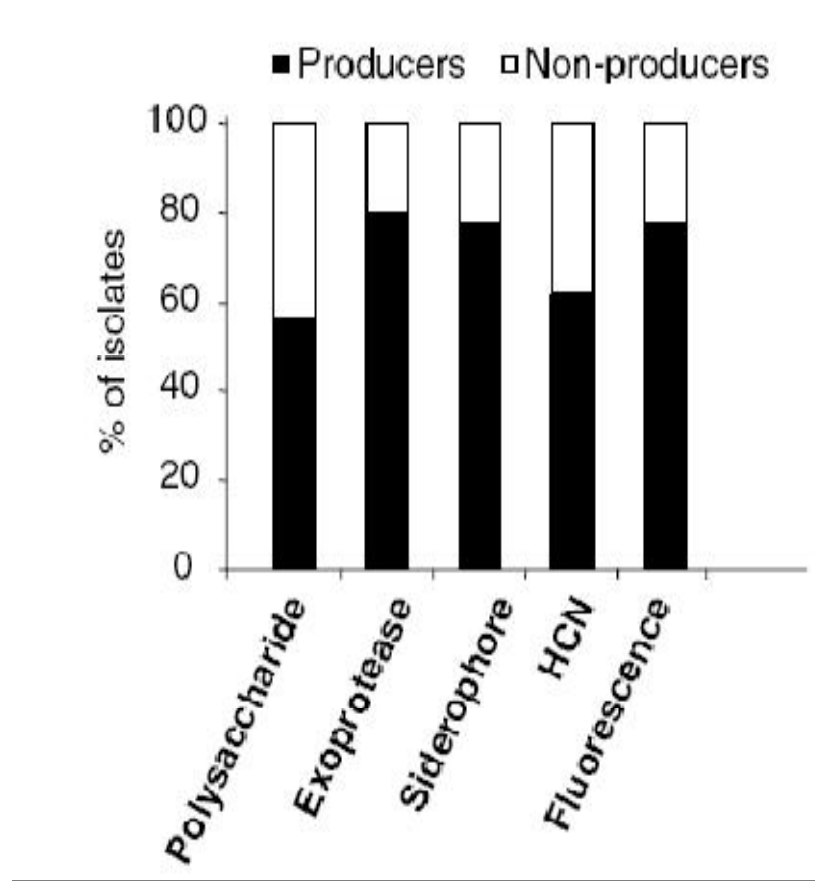
**Figure 2.2** Percentage of *C. elegans* lethal isolates isolated from water, frozen herbarium plant samples, dried herbarium plant samples and soil. Number in parenthesis indicates number of bacterial isolates tested. Bacteria identified from 16S rDNA sequence.



**Figure 2.3** Percentage of *C. elegans*-lethal isolates (total 63) that inhibit growth of *E. coli*, *A. rhizogenes*, *B. cereus*, *F. solani* and *M. incognita*.



**Figure 2.4** *In planta* bioassay using soybean cyst nematode on soybean seedlings. Graph represents two separate trials. The number of cyst per gram of roots was calculated. Data normalized relative to control (mock-inoculated). Biocontrol strain Pf-5 (gray bar) used as negative control. (\*) indicates significant difference from controls using student t-test ( $P < 0.05$ ).



**Figure 2.5** Percentage of *C. elegans*-lethal isolates (total 63) that produce exopolysaccharides, exoproteases, siderophores, hydrogen cyanide (HCN) and fluorescent compounds.



	<i>Motility</i>	<i>Mucoid</i>	<i>Exoprotease</i>	<i>Siderophore</i>	<i>HCN</i>	<i>Fluorescence</i>	<i>E. coli act.</i>	<i>A. rhizogenes act.</i>	<i>B. cereus act.</i>	<i>F. solani act.</i>	<i>RKN act.</i>
<i>Motility</i>		0.550	0.142	0.056	0.757	0.310	0.026	0.845	0.907	0.832	0.952
<i>Mucoid</i>	0.550		0.724	0.248	0.792	0.077	0.129	0.345	0.221	0.511	0.534
<i>Exoprotease</i>	0.142	0.724		0.939	0.011	0.186	0.161	0.694	0.061	0.469	0.164
<i>Siderophore</i>	0.056	0.248	0.939		0.460	0.828	0.003	0.000	0.143	0.283	0.041
<i>HCN</i>	0.757	0.792	0.011	0.460		0.024	0.343	0.350	0.110	0.047	0.397
<i>Fluorescence</i>	0.310	0.077	0.186	0.828	0.024		0.558	0.165	0.139	0.817	0.773
<i>E. coli act.</i>	0.026	0.129	0.161	0.003	0.343	0.558		0.002	0.175	0.808	0.097
<i>A. rhizogenes act.</i>	0.845	0.345	0.694	0.000	0.350	0.165	0.002		0.010	0.499	0.312
<i>B. cereus act.</i>	0.907	0.221	0.061	0.143	0.110	0.139	0.175	0.010		0.444	0.498
<i>F. solani act.</i>	0.832	0.511	0.469	0.283	0.047	0.817	0.808	0.499	0.444		0.421
<i>RKN act.</i>	0.952	0.534	0.163	0.041	0.397	0.773	0.097	0.312	0.498	0.421	

**Figure 2.6** Pair-wise correlation was performed using Pearson's correlation coefficient analysis. Traits comparisons with P-values less than 0.05 are likely correlated.

**Table 2.1:** *Pseudomonas* isolates examined

Bacteria ID	Source	<i>C. elegans</i> Activity	Motility	Polysaccharide producers	Exoprotease producers	Siderophore producers	HCN (qualitative)	HCN (quantitative)	Fluorescence	Inhibits <i>E. coli</i> growth	Inhibits <i>A. rhizogenes</i>	Inhibits <i>B. cereus</i>	inhibits <i>F.solani</i>	Lethal to (RKN)
1 B1	MS-R	Fast	1	Yes	Yes	1	3	7.0 ± 0.07	Blue Green	No	Yes	Yes	No	No
1 C5	MS-R	Fast	0	No	No	2	0	4.3 ± 0.02	Blue	No	Yes	Yes	Yes	No
1 F2	MS-R	Fast	2	Yes	Yes	2	3	280.1 ± 2.11	Blue	No	No	Yes	Yes	No
12 H11	MO-R	Fast	2	No	Yes	3	2	5.3 ± 0.05	Blue Green	Yes	Yes	Yes	Yes	No
14 B11	MO-R	Fast	1	No	Yes	0	3	328.7 ± 0.54	Orange	No	No	Yes	Yes	Yes
14 B2	MO-R	Fast	1	No	Yes	1	3	249.6 ± 7.05	Blue	No	Yes	Yes	Yes	Yes
14 D6	MS-R	Medium	2	No	No	3	0	4.0 ± 0.04	Blue Green	Yes	Yes	No	No	No
14 E2	MO-R	Fast	2	Yes	Yes	2	1	294.8 ± 1.35	Blue Green	No	Yes	Yes	No	No
14 E8	MO-R	Fast	2	Yes	Yes	3	0	241.8 ± 0.73	None	Yes	Yes	Yes	No	No
15 D11	MS-R	Fast	1	No	Yes	1	1	373.6 ± 3.26	Violet	No	No	Yes	No	No
15 G2	MO-R	Fast	2	Yes	Yes	3	3	15.2 ± 1.02	Blue	No	Yes	Yes	No	Yes
15 G6	MO-R	Fast	2	No	Yes	3	2	14.0 ± 0.67	Violet Blue	Yes	Yes	Yes	No	No
15 H10	MO-R	Fast	2	No	Yes	2	3	184.8 ± 0.87	Blue	Yes	Yes	Yes	Yes	No
15 H3	MO-R	Fast	2	No	Yes	2	3	18.3 ± 1.42	Blue	No	No	Yes	Yes	No
19 H3	MS-R	Fast	2	No	Yes	3	0	14.5 ± 1.32	None	No	No	No	Yes	No
2 F9	MO-R	Fast	2	Yes	Yes	3	3	148.6 ± 15.02	Blue Green	No	No	Yes	No	No

<b>22 A3</b>	Her	Medium	2	Yes	No	0	0	259.4 ± 1.29	None	No	No	No	Yes	Yes
<b>22 C4</b>	Her	Slow	2	Yes	No	0	0	314.3 ± 0.44	None	No	No	No	Yes	Yes
<b>24 D2</b>	Her	Slow	2	Yes	Yes	0	0	322.5 ± 0.53	Blue Green	No	No	Yes	No	No
<b>24 D3</b>	Her	Slow	2	Yes	No	1	3	75.4 ± 5.46	Blue Green	No	No	Yes	Yes	No
<b>25 C11</b>	Her	Fast	2	Yes	Yes	2	0	302.5 ± 1.97	None	Yes	No	No	No	No
<b>28 B5</b>	Her	Slow	2	Yes	Yes	3	1	0.7 ± 0.09	Blue Green	No	No	No	No	Yes
<b>29 G9</b>	Her	Fast	2	No	Yes	3	0	5.4 ± 0.04	Green	No	Yes	Yes	No	No
<b>3 D6</b>	MO-R	Fast	2	No	Yes	2	3	1.6 ± 0.07	Blue Green	Yes	Yes	Yes	Yes	No
<b>31 D2</b>	Her	Slow	2	No	No	1	0	2.0 ± 0.10	Blue Green	No	Yes	No	No	No
<b>36 B3</b>	Wy-S	Fast	2	Yes	Yes	1	3	371.2 ± 1.31	Blue Green	No	No	No	Yes	No
<b>36 B7</b>	Wy-S	Fast	0	No	Yes	0	3	341.6 ± 0.55	Blue	No	No	No	No	No
<b>36 C6</b>	Wy-S	Slow	1	Yes	Yes	3	0	1.8 ± 0.11	None	Yes	Yes	Yes	Yes	No
<b>36 C8</b>	Wy-S	Medium	2	No	No	3	3	113.6 ± 4.97	None	Yes	No	No	No	No
<b>36 D4</b>	Wy-S	Slow	0	No	Yes	0	3	91.4 ± 7.47	Blue	No	Yes	No	No	No
<b>36 F3</b>	Wy-S	Slow	2	Yes	Yes	3	3	2.0 ± 0.11	Blue Green	No	No	Yes	No	No
<b>36 G2</b>	Wy-S	Fast	2	Yes	Yes	2	3	0.3 ± 0.02	Blue Green	No	No	No	Yes	No
<b>37 A10</b>	Wy-S	Fast	1	Yes	Yes	3	1	0.9 ± 0.05	Blue Green	No	Yes	No	No	No
<b>37 A11</b>	Wy-S	Medium	1	Yes	No	2	0	354.9 ± 0.57	Blue Green	Yes	Yes	Yes	No	Yes
<b>37 D10</b>	Wy-S	Fast	1	No	No	3	0	385.7 ± 0.31	Blue	Yes	Yes	Yes	No	No
<b>37 F8</b>	Wy-S	Fast	2	Yes	No	3	0	419.2 ± 1.21	None	No	No	No	No	Yes

<b>38 A4</b>	Wy-S	Slow	1	Yes	Yes	3	1	0.7 ± 0.01	Blue Green	No	Yes	No	No	No
<b>38 D4</b>	Wy-S	Slow	0	No	Yes	2	0	0.2 ± 0.01	None	No	Yes	Yes	No	No
<b>38 D5</b>	Wy-S	Slow	2	No	Yes	2	0	0.4 ± 0.0	Violet Blue	No	No	No	Yes	No
<b>38 D7</b>	Wy-S	Slow	0	Yes	Yes	0	0	0.2 ± 0.01	None	No	No	No	No	No
<b>38 E5</b>	Wy-S	Slow	1	Yes	Yes	3	2	359.5 ± 1.82	Violet Blue	No	Yes	No	No	No
<b>38 F7</b>	Wy-S	Slow	1	Yes	No	0	2	433.1 ± 0.69	Blue Green	No	No	No	No	No
<b>38 G2</b>	Wy-S	Fast	1	Yes	Yes	2	0	12.5 ± 0.55	Blue Green	No	No	Yes	No	No
<b>39 A2</b>	Wy-S	Slow	1	Yes	No	3	1	0.5 ± 0.07	Blue Green	No	Yes	No	No	Yes
<b>48 B8</b>	Wis-S	Medium	1	No	Yes	0	3	486.0 ± 0.38	Orange	No	No	Yes	No	Yes
<b>48 C10</b>	Wis-S	Medium	1	Yes	Yes	2	1	1.6 ± 0.18	Orange	No	No	No	Yes	Yes
<b>48 D1</b>	Wis-S	Slow	0	Yes	Yes	3	2	0.4 ± 0.03	Blue Green	No	Yes	Yes	Yes	Yes
<b>48 D5</b>	Wis-S	Fast	2	Yes	Yes	3	3	5.3 ± 0.06	Blue Green	No	Yes	Yes	No	No
<b>48 G9</b>	Wis-S	Medium	1	No	Yes	0	3	285.5 ± 4.01	Orange	No	No	No	Yes	No
<b>48 H11</b>	Wis-S	Medium	1	No	Yes	1	2	6.3 ± 0.02	Orange	No	Yes	Yes	Yes	Yes
<b>528C10</b>	MO-R	Fast	0	No	Yes	0	0	6.2 ± 0.04	Blue	No	No	No	No	Yes
<b>528H7</b>	MO-R	Fast	0	Yes	Yes	0	3	52.4 ± 1.69	None	No	No	No	Yes	No
<b>88 A6</b>	MO-S	Slow	1	No	Yes	0	0	357.4 ± 0.77	Green	No	No	No	No	Yes
<b>88 B11</b>	MO-S	Medium	2	Yes	No	1	0	359.5 ± 1.82	None	No	No	No	No	Yes
<b>89 F1</b>	MO-S	Slow	1	Yes	Yes	1	3	274.8 ± 4.37	Blue Green	No	No	No	No	No
<b>90 D7A</b>	MO-S	Slow	1	Yes	Yes	1	3	1.0 ± 0.0	Violet Blue	No	No	Yes	Yes	Yes

<b>90 F12</b>	MO-S	Slow	1	Yes	Yes	1	3	0.2 ± 0.0	Violet Blue	No	No	No	Yes	Yes
<b>91 D1</b>	MO-S	Slow	2	No	Yes	0	0	0.6 ± 0.01	Violet	No	No	Yes	No	Yes
<b>93 D8</b>	MO-S	Fast	0	No	Yes	2	3	8.4 ± 1.38	Orange Yellow	No	No	Yes	Yes	No
<b>93 F8</b>	MO-S	Fast	0	Yes	Yes	2	3	0.7 ± 0.01	Orange Yellow	No	No	Yes	No	No
<b>93 G8</b>	MO-S	Medium	0	No	Yes	2	3	4.2 ± 0.09	Orange Yellow	No	No	Yes	No	No
<b>94 A2</b>	MO-S	Medium	0	Yes	Yes	3	0	4.1 ± 0.63	None	No	No	No	No	No
<b>94 G2</b>	MO-S	Slow	0	Yes	Yes	2	0	0.1 ± 0.0	None	No	Yes	Yes	Yes	No
<b>Pf mvp 1-4</b>		Fast	1	Yes	Yes	2	1	0.36 ± 0.0	Violet	No	Yes	Yes	No	No
<b>Pf Q2-87</b>		Fast	1	No	Yes	2	1	3.43 ± 0.0	None	Yes	Yes	Yes	No	No
<b>Pf5</b>		Fast	1	No	Yes	3	1	38.2 ± 0.0	Blue Green	Yes	Yes	Yes	Yes	No

## **CHAPTER III**

**Hydrogen cyanide produced by *Pseudomonas* sp. strain**

**15G2 contributes towards nematode lethality**

## Contribution of Authorship

All experiments, figures, figure legends, and Materials and Methods selection were conducted or created by Haytham Aly. The strain 15G2 transposon mutants were created by Dr. Joseph Kamalay and further characterization and analysis were done by Haytham Aly.

### 3.1 Introduction

Bacteria from the genus *Pseudomonas* have been extensively investigated for their biocontrol activities against plant pathogens (Cook and Baker, 1983; Cook et al., 1995; O'Sullivan and O'Gara, 1992; Weller et al., 2002; Zuckerman and Esnard, 1994). They are excellent colonizers of the rhizosphere (Lugtenberg et al., 2001), have positive effects on plant growth (Haas and Defago, 2005), and produce a wide range of secondary metabolites that play an important role in the suppression of soil-borne plant diseases (Banger and Thomashow, 1999; Haas and Defago, 2005). Certain strains of *Pseudomonas* produce small molecules, called siderophores, which chelate and sequester iron in the rhizosphere, preventing its availability to other organisms (Haas and Defago, 2005; Thomashow and Weller, 1996). Examples of the well-characterized secondary metabolites are: 2,4-diacetylphloroglucinol (DAPG), phenazine-1-carboxylic acid (PCA), phenazine-1-carboxamide (PCN, also known as chlororaphin), pyoluteorin (PLT), pyrrolnitrin (PRN), extracellular protease and hydrogen cyanide (HCN) (Blumer and Haas, 2000b; Haas and Keel, 2003; Hammer et al., 1997; Kraus and Loper, 1995; Siddiqui et al., 2005). Most biocontrol *Pseudomonas* produce one or more of these secondary metabolites that account for their biocontrol activities.

Hydrogen cyanide is widely generated among the *Pseudomonas* spp. including many isolates of *P. fluorescens*, *P. aeruginosa*, *P. aureofaciens*, and *P. chlororaphis* under O<sub>2</sub>-limiting conditions (Blumer and Haas, 2000a). In *P. aeruginosa*, hydrogen cyanide is the primary virulence factor responsible for the lethality toward the nematode, *Caenorhabditis elegans* (Darby et al., 1999; Gallagher and Manoil, 2001), and *Drosophila melanogaster* (Broderick et al., 2008). HCN produced by *P. fluorescens* CHA0 contributes to the biocontrol activity against the fungal black root-rot disease (Laville et al., 1998; Voisard et al., 1989) and *Meloidogyne* spp. (Siddiqui and Shaukat, 2002; Siddiqui et al., 2006).

Hydrogen cyanide production in *Pseudomonas* spp. is catalyzed by a membrane-bound flavoenzyme called HCN synthase. This enzyme oxidizes glycine to produce HCN and carbon dioxide (Wissing, 1974; Freeman et al., 1975; Castric, 1977; Askeland and Morrison, 1983). HCN synthase has been partially purified from *Pseudomonas* species but the structure is still to be determined (Blumer and Haas, 2000). Cyanogenesis in *Pseudomonas* spp. is affected by oxygen levels, iron availability and cell density. The highest expression of HCN synthase and, thus, HCN production occurs under low oxygen concentrations (Castric, 1983; Castric, 1994) and during the transition from exponential to the stationary phase (Castric et al., 1979). Depletion of iron from *P. fluorescens* CHA0 cultures can also abolish HCN production (Blumer and Haas, 2000). In fluorescent *Pseudomonas*, HCN synthases are encoded by three structural genes, *hcnA*, *hcnB*, and *hcnC*, which form an operon (Laville et al., 1998). Although little is known about HCN synthases, their protein sequences share similarities with well known dehydrogenases and oxidases (Blumer and Haas, 2000). HCN production in most



*Pseudomonas* strains is regulated by the two-component system GacS/GacA (Laville et al., 1992; Reimann et al., 1997) and requires an anaerobic regulator of arginine deiminase and nitrate reductase (ANR). The anaerobic regulator *anr* mutants of *P. aeruginosa* produce small amounts of HCN compared to wild type strain (Zimmermann et al., 1991).

The molecular targets for hydrogen cyanide in other organisms are mainly cytochrome-c oxidase and metalloenzymes, leading to irreversible blockage of the mitochondrial aerobic respiration (Way, 1984; Blumer and Haas, 2000). Some *Pseudomonas* species possess cyanide-resistant alternative terminal oxidases that can avoid cyanide inhibition and drive cell respiration (Junemann, 1997). For example *P. pseudoalcaligenes* CECT5344 possesses two cyanide-insensitive oxidases (*cioA* and *cioB*) that allow the bacteria to grow and use the cyanide as the sole nitrogen source (Quesada et al., 2007). We chose one of our *Pseudomonas* strains for further characterization. *Pseudomonas* strain 15G2 was chosen for further analysis because: 1) This strain possesses activities against several plant pathogens such as *Agrobacterium rhizogenes* and plant-parasitic nematodes, 2) it is a motile bacterium that may actually represent an important fitness trait for studying the activity against migratory stages of nematodes and/or other phytopathogens, and 3) the strain possesses several biochemical characteristics such as production of exoproteases, siderophores, polysaccharides, DAPG and HCN commonly seen in many *Pseudomonas* used as biocontrol agents.

The aim of this study is to determine the role of HCN produced by *Pseudomonas* sp. strain 15G2 in the biological control of soybean cyst nematodes.

## 3.2 Materials and Methods

### 3.2.1 Bacterial and nematode strains, plasmids, culture conditions

Bacterial and nematode strains, and plasmids used in this study are listed in Table 3.1. Strains of *Pseudomonas* and *Escherichia coli* were routinely grown on King's medium B (KMB) (King et al., 1954) and Luria-Bertani (LB) medium (Invitrogen Corp., Carlsbad, CA) at 30°C and 37°C, respectively. For hydrogen cyanide quantification, *Pseudomonas* strains were grown on LB broth with shaking at 200 rpm and under oxygen limitation as described in *Chapter II*. For determination of amino acid utilization by the wild-type strain 15G2 and Tn5 mutants (*16-C8*, *9-H12*, and *18-A1*), bacteria were grown overnight on M9 minimal medium (Sambrook et al., 1989) supplemented with 20 mM glucose and 20 mM NH<sub>4</sub>Cl. Then cells were washed and transferred to fresh M9 medium supplemented with 10 mM amino acids as the sole carbon and nitrogen source or to M9 medium supplemented with 20 mM glucose and 10 mM amino acids as the sole nitrogen source. Antibiotics were used when required at concentrations (micrograms per milliliter) indicated in the parentheses: rifampicin (100), chloramphenicol (25), tetracycline (30) and ampicilin (100).

The *Caenorhabditis elegans* strains used in this study were maintained on nematode growth media (NGM) (Lewis and Fleming, 1995). The root-knot nematode, *Meloidogyne incognita*, was maintained on *Arabidopsis* plants according to Hammes et al. (2005). The soybean cyst nematode, *Heterodera glycines*, was maintained on soybean plants in sand in the greenhouse.

**Table 3.1** Bacterial, nematode strains and plasmids

Strain or Plasmid	Genotype or Phenotype*	Reference or source
<b>Strains</b>		
<b><i>Pseudomonas</i></b>		
<i>P. fluorescens</i> -Pf-5	Wild type	Linda Thomashaw
15G2	Rif <sup>r</sup>	This Study
15G2-CGT 9033	<i>ΔhcnABC</i> ; Rif <sup>r</sup> ; Km <sup>r</sup>	This Study
15G2-CGT 9036	<i>ΔhcnA</i> ; Rif <sup>r</sup> ; Km <sup>r</sup>	This Study
15G2-CGT 9040	<i>ΔhcnB</i> ; Rif <sup>r</sup> ; Km <sup>r</sup>	This Study
15G2-CGT 9043	<i>ΔhcnC</i> ; Rif <sup>r</sup> ; Km <sup>r</sup>	This Study
15G2-CGT-4628	Expressing GFP	Chris Taylor
<b><i>E. coli</i></b>		
DH5α	<i>recA1 endA1 hsdR17 supE4 gyrA96 relA1 Δ(lacZYA-argF)U169 (Φ80dlacZΔM15)</i>	
<b>Nematodes</b>		
<i>H. glycines</i>	TN17	Terry Niblack
<i>M. incognita</i>		Chris Taylor
<i>C. elegans</i> -N2	Wild type	<i>Caenorhabditis</i> Genetics Center
<b>Plasmids</b>		
pAKK 1410	Shuttle vector; ColE replicon; Ap <sup>r</sup>	This Study
pCGT 4628	pAKK 1410 with 0.88kb <i>HindIII-SacI</i> fragment containing GFP behind <i>Bacillus</i> promoter	This Study
pCGT 9004	pAKK 1417 with 0.9kb <i>KpnI-SacI</i> fragment containing GFP driven by <i>Bacillus</i> promoter	This Study
pCGT 4632A	Broad-host range vector; RK2 replicon; Tet <sup>r</sup> ; Km <sup>r</sup>	
pCGT 9005A	pCGT 4632 with 0.9kb <i>pmeI</i> fragment containing GFP gene behind <i>Bacillus</i> promoter	This Study
pCGT 9010	pCGT 4628 with 1.67kb <i>NotI</i> fragment containing 16C8 gene behind <i>Bacillus</i> promoter	This Study
pCGT 9011	pCGT 4628 with 1.67kb <i>NotI</i> fragment containing 9H12 gene behind <i>Bacillus</i> promoter	This Study
pCGT 9012	pCGT 4628 with 1.67kb <i>NotI</i> fragment containing 18A1 gene behind <i>Bacillus</i> promoter	This Study
pCGT 9013A	pCGT 9005A with 1.67kb <i>NotI</i> fragment containing 16C8 gene behind <i>Bacillus</i> promoter	This Study
pCGT 9014A	pCGT 9005A with 1.67kb <i>NotI</i> fragment containing 9H12 gene behind <i>Bacillus</i> promoter	This Study
pCGT 9015A	pCGT 9005A with 1.67kb <i>NotI</i> fragment containing 18A1 gene behind <i>Bacillus</i> promoter	This Study
pCGT 4441	Suicide vector; OriT; ColE1 replicon; Cam <sup>r</sup>	
pCGT 4433B	Cloning vector; ColE1 replicon; Ap <sup>r</sup> ; Km <sup>r</sup>	This Study
pCGT 9030	pCGT 4433B with 0.94kb <i>BamHI-EcoRI</i> fragment containing <i>hcnABC</i> -5' flanking sequences	This Study
pCGT 9031	pCGT 4433B with 1.04kb <i>ClaI-Acc65I</i> fragment containing <i>hcnABC</i> -3' flanking sequences	This Study
pCGT 9032	pCGT 9030 with 1.04kb <i>ClaI-Acc65I</i> fragment containing <i>hcnABC</i> -5' flanking sequences.	This Study

**Table 3.1** Bacterial, nematode strains and plasmids (Continued)

Strain or Plasmid	Genotype or Phenotype*	Reference or source
<b>Plasmids</b>		
pCGT 9033	pCGT 4441 with 3.7kb <i>PacI</i> fragment containing <i>hcnABC</i> -5' -Km <sup>r</sup> gene- <i>hcnABC</i> -3' cassette	This Study
pCGT 9034	pCGT 4433B with 1.41kb <i>ClaI</i> - <i>Acc65I</i> fragment containing <i>hcnB</i> gene	This Study
pCGT 9035	pCGT 9030 with 1.41kb <i>ClaI</i> - <i>Acc65I</i> fragment containing <i>hcnB</i> gene	This Study
pCGT 9036	pCGT 4441 with 4.06kb <i>PacI</i> fragment containing <i>hcnABC</i> -5' -Km <sup>r</sup> gene- <i>hcnB</i> gene cassette	This Study
pCGT 9037	pCGT 4433B with 1.23kb <i>Bam</i> HI- <i>Eco</i> RI fragment containing <i>hcnB</i> -5' flanking sequences	This Study
pCGT 9038	pCGT 4433B with 1.25kb <i>ClaI</i> - <i>Acc65I</i> fragment containing <i>hcnC</i> gene	This Study
pCGT 9039	pCGT 9037 with 1.23kb <i>ClaI</i> - <i>Acc65I</i> fragment containing <i>hcnC</i> gene	This Study
pCGT 9040	pCGT 4441 with 4.2kb <i>PacI</i> fragment containing <i>hcnB</i> -5' -Km <sup>r</sup> gene- <i>hcnC</i> gene cassette	This Study
pCGT 9041	pCGT 4433B with 1.41kb <i>Bam</i> HI- <i>Eco</i> RI fragment containing <i>hcnB</i> gene	This Study
pCGT 9042	pCGT 9031 with 1.41kb <i>Bam</i> HI- <i>Eco</i> RI fragment containing <i>hcnB</i> gene	This Study
pCGT 9043	pCGT 4441 with 4.18kb <i>PacI</i> fragment containing <i>hcnB</i> gene -Km <sup>r</sup> gene- <i>hcnABC</i> -3' gene cassette	This Study

\* Tet<sup>r</sup>, tetracycline resistance; Km<sup>r</sup>, kanamycin resistance; Ap<sup>r</sup>, ampicillin resistance; Rif<sup>r</sup>, rifampicin resistance.

### 3.2.2 Transposon mutagenesis

**Generating transposon mutants.** *Pseudomonas* sp. strain 15G2 mutant libraries were constructed using the EZ::TN<sup>TM</sup> <R6Kγori/KAN-2> Transposome kit (Epicentre Biotechnologies, Madison, WI, USA) according to the manufacturer's instructions. Electrocompetent strain 15G2 cells were prepared according to a protocol (Farinha and Kropinski, 1990) modified by addition of 0.5% threonine to the cell growth medium. Following electroporation, cells were grown at 30°C on LB plates supplemented with kanamycin, and the kanamycin resistant colonies were isolated. Colonies were picked and placed into a 96-deep well microtiter plate containing Kings-B media and grown overnight.

**Mutant screening.** To screen for non-nematode lethal mutants, colonies were screened against *C. elegans* as previously described in *Chapter II*. Mutants with loss (or knock out) of nematode lethality were retested in triplicate.

**DNA sequencing.** To determine the chromosomal location of the inserted transposon, genomic DNA was extracted from those Tn-5 mutants that lost their nematode-lethality. The DNA was digested with restriction enzyme, *Nco* I, diluted, and religated to generate small circular pieces of DNA (because inserts contained a kanamycin selectable marker and an *E. coli* origin of replication). Ligated DNA was electroporated into *pir*<sup>+</sup> *E. coli* cells, provided with the transposon kit, and grown on kanamycin-LB plates. Kanamycin resistant colonies were picked and grown overnight. Rescued plasmids were sequenced using transposon specific primers. DNA sequences

flanking the transposon were identified by BLASTN searches of *Pseudomonas* genomic sequences.

### **3.2.3 Phenotypic characterization assays**

Phenotypic characterization of *Pseudomonas* sp. strain 15G2 including: motility, exoprotease activity, siderophore and polysaccharide production, and qualitative assays for hydrogen cyanide production were performed as described in *Chapter II*.

### **3.2.4 Root attachment assay**

Root attachment assay was performed according to Turnbull et al. (2001) with minor modifications. Seven-day-old soybean seedlings growing on sterilized sand were taken out and washed with sterile water and incubated individually with 5 mL of either GFP-tagged *Pseudomonas* sp. strain 15G2 or GFP-tagged *E. coli* cultures with OD<sub>600</sub> adjusted to 0.3. The roots with the bacterial suspensions were then incubated at room temperature for 2 hours under gentle agitation. After incubation, the roots were washed five times by vigorous shaking in sterile water to remove any nonattached or weakly attached bacteria. Plant roots were sectioned to 20-30  $\mu$ M thick slices and placed on a microscope slide. Aggregations of fluorescent bacterial cells on plant roots were visualized by confocal scanning laser microscope (CSLM, Leica Model TCS, Heidelberg, Germany).

### **3.2.5 Quantification of bacterial cyanide production**

For wild-type strain 15G2,  *$\Delta$ hcn* and *Tn-5* mutants, hydrogen cyanide production was measured as described in *Chapter II* using a micro-cyanide-ion selective electrode

(Lazar Research Laboratories, Los Angeles, CA, USA), except that HCN measurements were conducted after three days of culturing the bacteria.

### **3.2.6 Exposure of *C. elegans* to bacterial cyanide**

Nematode paralytic killing assays were carried out according to Gallagher and Manoil (2001) with some modifications. Briefly, LB plates supplemented with 12.5 mM glycine were inoculated with either WT-15G2 strain or 15G2- Tn-5 knock-outs (A1, G3, and 18E3) and incubated at 30°C overnight. LB plates without bacteria or inoculated with *E. coli* DH5 $\alpha$  were used as negative controls. *C. elegans* from stock plates were collected in S-full medium, and 100  $\mu$ l aliquots (containing 50-75 worms) were placed in 3.5-cm-diameter plates. Each bacterial culture plate was left open with the opened plate containing the nematodes in a sealed 10-cm-diameter plate. The nematode was deemed dead if it failed to move or respond to tapping the plate against the microscope stage. The number of dead nematodes was recorded every two hours. Similar experiments were done using freshly hatched *M. incognita* J2s instead of *C. elegans*. Data represents three individual biological replicates.

### **3.2.7 DNA manipulation and nucleotide sequencing**

*Pseudomonas* genomic DNA was extracted with a genomic DNA purification system (Wizard SV; Promega Corp, Madison, WI) according to the manufacturer's instructions. Plasmid DNA isolation and purification from *E. coli* were carried out using PureYield™ Plasmid Midiprep System (Promega Corp, Madison, WI) according to the manufacturer's instructions. Restriction enzyme digestion, gel electrophoresis, and *E. coli* transformation were performed using standard techniques (Sambrook et al., 1989).

PCR and digestion reactions were purified using QIAquick PCR Purification and QIAquick Gel Extraction Kit, respectively (Qiagen Valencia, CA). The pCR2.1 TA and Zero Blunt TOPO cloning vectors (Invitrogen, Carlsbad, Calif.) were used to clone PCR products according to the manufacturer's instructions. Primers used in this study were designed using Vector NTI software (Invitrogen Corp., Carlsbad, Calif.) and introduced restriction sites were underlined (Table 3.2). PCR reactions were carried out following standard protocols unless otherwise indicated. For nucleotide sequence determination, cloned genes or PCR fragments were sent to The Genome Sequencing Center at Washington University School of Medicine in St. Louis, Missouri. The sequence data were compiled and analyzed with the Vector NTI software (Invitrogen Corp., Carlsbad, Calif.). Database searches were performed by NCBI's BLAST network and *Pseudomonas* Genome database server (<http://www.pseudomonas.com/>).

### **3.2.8 *Pseudomonas* sp. strain 15G2-*hcnABC* sequence analysis**

**PCR primer design.** The *hcnABC* nucleotide sequences of *P. fluorescens* strain Pf-5 and strain Pf0-1, *P. entomophila* L48, *P. aeruginosa* PAO1, and *P. aeruginosa* PA14 were obtained from the *Pseudomonas* Genome database and aligned using the Vector NTI software (Invitrogen Corp., Carlsbad, Calif.). Based on sequence similarities, two sets of primers were designed: BWZ and CIA (Table 3.2) to amplify 650 bp of *hcnB* gene, and CIB and CIC (Table 3.2) to amplify 700 bp of *hcnC* gene.

**PCR amplification of *hcnB* and *hcnC*.** PCR amplification reactions for *hcnB* and *hcnC* were carried out in 50  $\mu$ L reaction mixtures containing 100 ng of 15G2-genomic DNA as a template, 1X Phusion HF buffer, 200  $\mu$ M each of dATP, dCTP, dGTP



and dTTP, 0.5  $\mu$ M of each primer, and 1U of Phusion DNA Polymerase (New England BioLabs, USA). Thermal cycling condition consisted of initial denaturation (at 98°C for 1 min), followed by 35 cycles of 98°C for 10 sec, 58°C for 30 sec, and 72°C for 30 sec,, and a final cycle at 72°C for 10 min. The PCR products were visualized on 1.5% agarose gels. The expected size bands were excised and cloned into Zero Blunt TOPO vector and transformed into electrocompetent *E. coli* strain DH5 $\alpha$ . The transformants were screened and plasmids were sent for sequencing using M13F and M13R primers.

#### **Determining 15G2-*hcnABC* sequence and their 5' and 3' flanking sequences.**

In order to obtain the full sequence of the 15G2-*hcnABC* and their 5' and 3' flanking sequences, self-formed adaptor PCR (SEFA PCR) method was used (Wang et al., 2007). To determine the sequences flanking the 15G2-*hcnB* partial sequence, we obtained three primers. DGE, DGF, and DGI (Table 3.2) were designed to be located sequentially on the same strand in the same direction. Also, another three primers, DGJ, DGK, and DGN (Table 3.2), designed similarly on the 15G2-*hcnC* partial sequence, were used to determine the flanking sequences. PCR reactions and conditions were carried out according to Wang et al. (2007).

### **3.2.9 Plasmid constructions**

#### **3.2.9.1 Construction of 15G2- *hcn* mutants by gene replacement**

To construct the chromosomal  $\Delta$ *hcnABC* mutant (15G2- $\Delta$ *hcnABC*, Figure 3.1), the *hcnABC* flanking sequences were amplified by PCR using 15G2 genomic DNA as a template. 936 bp of the 5' flanking sequence was amplified using DGS and DGT primers, and 1000 bp of the 3' flanking sequence was amplified using DGU and DGV primers. The amplification reactions and thermal cycling conditions were as described

in section 3.2.8. The resulting fragments were then cloned into the shuttle vector pCGT 4433B to create the pCGT 9030 and pCGT 9031 vectors, respectively (Table 3.1). These *hcnABC* flanking sequences were then cloned bordering the kanamycin resistance gene into the suicide vector pCGT 4441, which carries the *sacB/sacR* negative selection marker (Jackson et al., 2001), to result in pCGT 9033 (Table 3.1). To obtain other *hcn* mutant strains: 15G2- $\Delta hcnA$ , 15G2- $\Delta hcnB$ , 15G2- $\Delta hcnC$  (Figure 3.1), a cloning strategy similar to those explained above was used. Primers used for amplification of flanking sequences are listed in Table 3.2 and the resulting vectors which carry the gene deletion are listed in Table 3.1. After the construction of the deletion derivatives pCGT 9033, pCGT 9036, pCGT 9040, and pCGT 9043, the suicide plasmids were then transformed into electrocompetent 15G2 cells and allowed to chromosomally integrate. Integration events (first crossover) were selected by plating the bacteria on LB supplemented with kanamycin. Screening for the excision of vectors by a second crossover was carried out by plating the cells on LB supplemented with kanamycin and 15% sucrose. Only those colonies that were resistant to growth on 15% sucrose and kanamycin were selected for PCR confirmation and DNA sequencing.

### 3.2.9.2 Construction of complemented mutants

Three 15G2-transposon mutants defective in their HCN production were chosen for complementation analysis. Full-length copies of *9-H12*, *16-C8*, and *18-A1* genes were cloned into the broad-host-range plasmid pCGT 4632A (Table 3.1). First, primers that allow the amplification of 9-H12, 16-C8, and 18-A1 genes (Table 3.2) were designed according to the *P. fluorescens* Pf-5 genome sequence obtained from the *Pseudomonas* database. All amplifications were carried out with Phusion DNA Polymerase according

to manufacturer's instructions (New England BioLabs, USA). The PCR products were then cloned into the shuttle vector pCGT 4628 behind a *Bacillus* constitutive promoter (previously isolated in the Taylor lab) to produce plasmids pCGT 9010 (16-C8), pCGT 9011 (9-H12), and pCGT 9012 (18-A1) (Table 3.1). These plasmids were sent for sequencing to confirm integrity and then transferred to the broad-host-range pCGT 4632A to result in pCGT 9013A (16-C8), pCGT 9014A (9-H12) and pCGT 9015A (18-A1) (Table 3.1). These plasmids were then electroporated into  $\Delta 16-C8$ ,  $\Delta 9-H12$ , and  $\Delta 18-A1$  mutants, and restoration of HCN production was assayed as described in *Chapter II*.

### 3.2.10 Southern blot analysis

Three micrograms of genomic DNA purified from WT-15G2, 15G2-*ΔhcnABC*, and *P. fluorescens* strain Pf-5 were cut with *EcoRI* and *NcoI* overnight at 37°C and run on 1% agarose gel. The gel was then denatured in a mixture of 0.5 M Tris-Cl and 1.5 M NaOH, neutralized, transferred onto Hybond N+ membrane (GE Healthcare Bio-Sciences Corp, Piscataway, NJ), and fixed by cross-linking the membrane with UV exposure. Primers BWZ and CIA (Table 3.2) were used to amplify 650 bp of *hcnB* gene to be used as a probe. For the amplification reaction, 100 ng of genomic DNA isolated from WT-15G2 strain (as a template) and the Phusion™ High-Fidelity DNA Polymerase (New England BioLabs Ipswich, MA) were used following the manufacturer's protocol. The resulting fragment was then cloned in the Zero Blunt TOPO vector. The probe was labeled and detected by means of an AlkaPhos Direct Labeling and Detection system (GE Healthcare Bio-Sciences Corp). Membranes were equilibrated in hybridization buffer (GE Healthcare Bio-Sciences Corp) for 2 hours and then hybridized at 65°C overnight in

the same buffer with 5  $\mu\text{L}/\text{mL}$  of labeled probe. After two high-stringency (primary wash buffer) and two low-stringency washes (secondary wash buffer), the labeled probe-DNA hybrid was visualized by luminescent detection technique according to the manufacturer's protocol.

### **3.2.11 *In vivo* lethality to soybean cyst nematodes.**

The *in planta* activity of the *Pseudomonas* sp. strain 15G2,  $\Delta hcnABC$ ,  $\Delta hcnA$ ,  $\Delta hcnB$ ,  $\Delta hcnC$ , and 15G2-Tn-5 mutants (E-1, 18-F6) against the soybean cyst nematode *H. glycines* was tested as described in *Chapter II*. This experiment was repeated three times.

### **3.2.12 Nucleotide sequence accession number**

The nucleotide sequence of the complete *hcnABC* operon of *Pseudomonas* sp. strain 15G2 is available under GenBank accession number GQ339615.

**Table 3.2** Primers designed in this study

Name	Target gene*	Primer sequence
<b>DGE</b>	5' SP1- <i>hcnB</i>	5'-TCGACCACGCCCCAGCCATAGCGCAGGG-3'
<b>DGF</b>	5' SP2- <i>hcnB</i>	5'-AGCATGCTCAGGCCGTCGAGAAACAG-3'
<b>DGI</b>	5' SP3- <i>hcnB</i>	5'-GCTCTGCTTGGCGATNNNNNNNNNGGCAC-3'
<b>DGJ</b>	3' SP1- <i>hcnC</i>	5'-ACCACCGAGGACAAGGGCTTCGACGTCA-3'
<b>DGK</b>	3' SP2- <i>hcnC</i>	5'-GTCAACCTCAAGCGCTGCTGGGCGGG-3'
<b>DGN</b>	3' SP3- <i>hcnC</i>	5'-ACCGGCATCCTGACNNNNNNNNNACCGGC-3'
<b>DGS</b>	5' <i>hcnABC</i> -F	5'- <u>CCC</u> <u>GGATCC</u> GGGATTCTAAATAAACTTTCATCTGGC-3'
<b>DGT</b>	5' <i>hcnABC</i> -R	5'- <u>CCC</u> <u>GAA</u> TCTTGC <del>GGT</del> TATCTGACGCATTGCGGTTC-3'
<b>DGU</b>	3' <i>hcnABC</i> -F	5'- <u>CCC</u> <u>ATCG</u> ATGTTGGACTTGC <del>GT</del> AGGAGCTGGCTTGCC-3'
<b>DGV</b>	3' <i>hcnABC</i> -R	5'- <u>CCC</u> <u>GGT</u> ACCTGCCGTTGC <del>CAG</del> CCTGCGGCAGCGGC-3'
<b>DGW</b>	<i>hcnB</i> -F	5'- <u>CCCC</u> <u>ATCG</u> ATATGAGCCTGAACCCGGTGATTGTCGG-3'
<b>DGX</b>	<i>hcnB</i> -R	5'- <u>CCC</u> <u>GGT</u> ACCTTACTCATGTCCGGGGACCTCCTGGTC-3'
<b>DGY</b>	5' <i>hcnB</i> -R	5'- <u>CCC</u> <u>GAA</u> TCTCATACCGTGGGCTCCGTCTCGCTGATG-3'
<b>DGZ</b>	<i>hcnC</i> -F	5'- <u>CCC</u> <u>ATCG</u> ATATGAGTAAGTATTACGACGTGGTGATTG-3'
<b>DHA</b>	<i>hcnC</i> -R	5'- <u>CCC</u> <u>GGT</u> ACCTCAGGCCGGTTCGGGCTGTTTCTTACC-3'
<b>DHB</b>	<i>hcnB</i> -F	5'- <u>CCC</u> <u>GGATCC</u> ATGAGCCTGAACCCGGTGATTGTCGG-3'
<b>DHC</b>	<i>hcnB</i> -R	5'- <u>CCC</u> <u>GAA</u> TCTTACTCATGTCCGGGGACCTCCTGGTC-3'
<b>DAA</b>	18- <i>A1</i> -F	5'- <u>CCC</u> <u>GGATCC</u> ATGAGCAAGACTTCTCTCGA-3'
<b>DAB</b>	18- <i>A1</i> -R	5'- <u>CCC</u> <u>GAGCTC</u> CGCGCCGCTTAGAACAGCACGCGGCAAC-3'
<b>DAC</b>	9- <i>H12</i> -F	5'- <u>CCC</u> <u>GGATCC</u> ATGAAAATGCATAAGACCCT-3'
<b>DAD</b>	9- <i>H12</i> -R	5'- <u>CCC</u> <u>GAGCTC</u> CGCGCCGCCTACAGGGCCGCGTCGATTT-3'
<b>DAE</b>	16- <i>C8</i> -F	5'- <u>CCC</u> <u>GGATCC</u> ATGAAGATGTTGAAATCCACCCTGG-3'
<b>DAF</b>	16- <i>C8</i> -R	5'- <u>CCC</u> <u>GAGCTC</u> CGCGCCGCTCAGCGCACAGGTGGTGCCTG-3'
<b>BWZ</b>	<i>hcnB</i> -F	5'-CTCGANGGCCTGAGCATGCTCG-3'
<b>CIA</b>	<i>hcnB</i> -R	5'-CGCCCCTGGCAGTCGCC CATGC-3'
<b>CIB</b>	<i>hcnC</i> -F	5'-TCGGTGGGCCTGGGCTGCGGTGT-3'
<b>CIC</b>	<i>hcnC</i> -R	5'-CTGCCAATCAGGATCTCGCC-3'

\* F, forward; R, reverse; underlined nucleotides are the restriction sites introduced.

### 3.3 Results

#### 3.3.1 Characterization of *Pseudomonas* sp. strain 15G2.

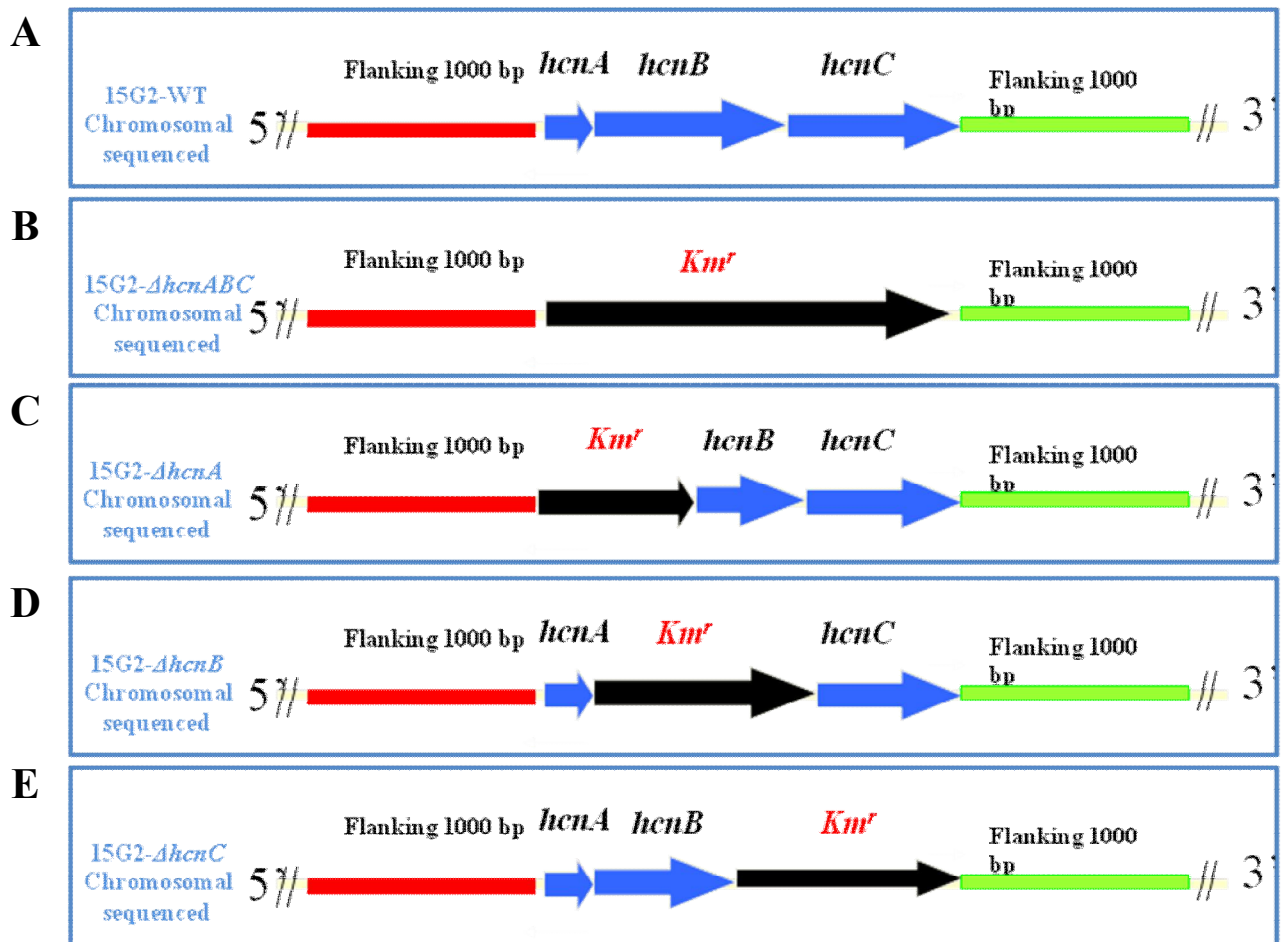
In order to determine the factors or characteristics that make the *Pseudomonas* sp. strain 15G2 a fast killer of the *C. elegans* L2s, several phenotypic assays were used. Our results showed that strain 15G2 was motile when grown on LB medium solidified with low agar concentrations (0.1-1%) and failed to swarm at a higher concentration (1.5%). Strain 15G2 was a blue siderophore producer when grown on CAS agar plates (Figure 3.2F) and was highly mucoid on *Pseudomonas* agar plates (Figure 3.2B). The extracellular protease activity was assayed on skim milk agar plates, and results showed the 15G2 strain's ability to metabolize the casein in the medium, which appeared as a clear halo around the bacterial growth area (Figure 3.2 A). In addition, this strain was a high HCN producer (Figure 3.2D) and contains the biosynthetic genes for the production of the antibiotic, 2,4-diacetylphloroglucinol (DAPG) (data from *Chapter II*). The ability of the strain to inhibit the growth of bacterial and fungal organisms was also tested. The growth of *Agrobacterium rhizogenes* and *Bacillus cereus* was inhibited by strain 15G2, whereas the growth of both *E. coli* and *Fusarium solani* was not affected (Figure 3.2 C). Results from *Chapter II* showed the potential activity of the strain against both root-knot and cyst nematodes *in vitro* and *in vivo*.

To determine the ability of the strain 15G2 to attach and colonize the plant roots, GFP-tagged strains of wild-type 15G2 cells and *E. coli* (control) were used. After two hours of root incubation with bacterial suspensions and repeatedly aggressive washes, the bacteria's attachment to the roots was visualized using confocal microscopy. Our results

showed that some GFP-15G2 cell aggregates were observed along the whole root surface and no attachment was observed for roots inoculated with *E. coli* (Figure 3.3).

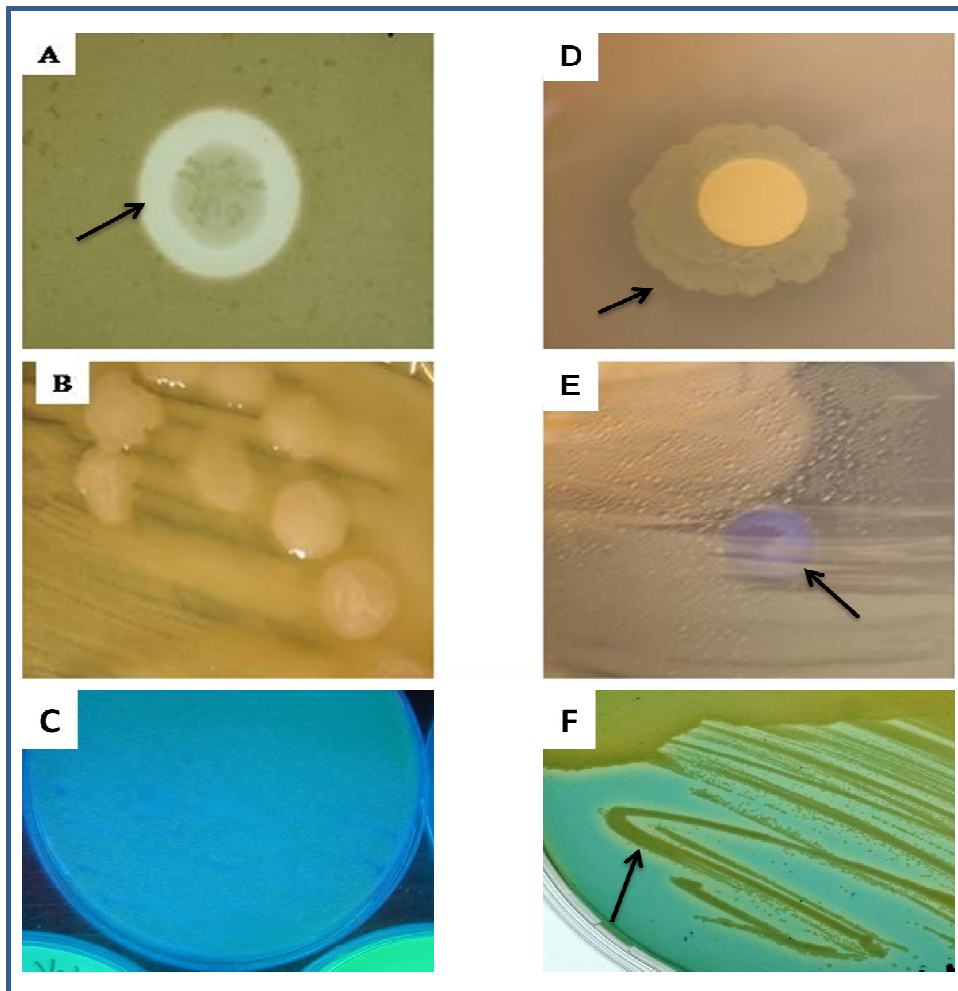
### **3.3.2 Production of HCN in *Pseudomonas* sp. strain 15G2 is cell density dependent**

Production of HCN by strain 15G2 was assayed quantitatively during different phases of bacterial growth under limited oxygen levels. Colony forming units (CFUs), absorbance (OD<sub>600nm</sub>), and HCN measurements were determined every two hours. The results demonstrate that HCN production started when the cells enter the exponential phase and maximum production occurred during the transition between the exponential to stationary growth phase (Figure 3.4). These results indicate that HCN production is strongly dependent on cell density. Since cyanide production is known to be stimulated by the addition of glycine to the medium (Wissing, 1974), a similar experiment to those described above was performed in LB medium supplemented with glycine. The results showed that glycine does not have noticeable effects on the growth rate or HCN production in strain 15G2.

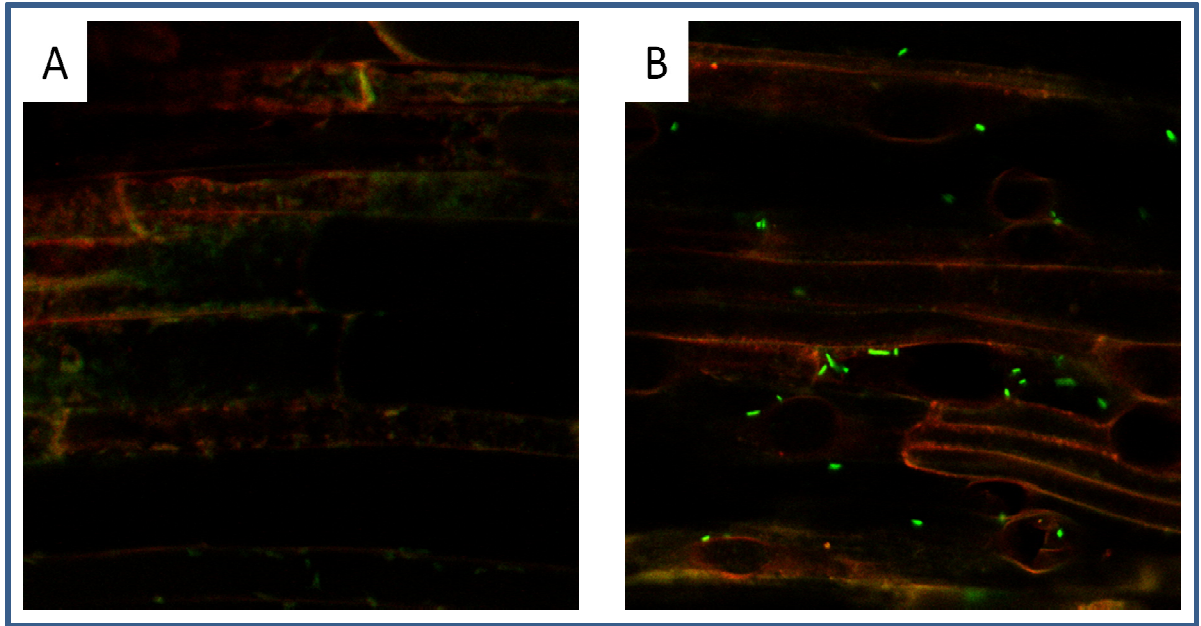


**Figure 3.1** Schematic representation of the chromosomal locus of the *hcnABC* gene cluster in wild type *Pseudomonas sp.* strain 15G2 (A), 15G2- $\Delta hcnABC$  (B), 15G2- $\Delta hcnA$  (C), 15G2- $\Delta hcnB$  (D), and 15G2- $\Delta hcnC$ . Symbols: *Km<sup>r</sup>*, kanamycin resistance gene.

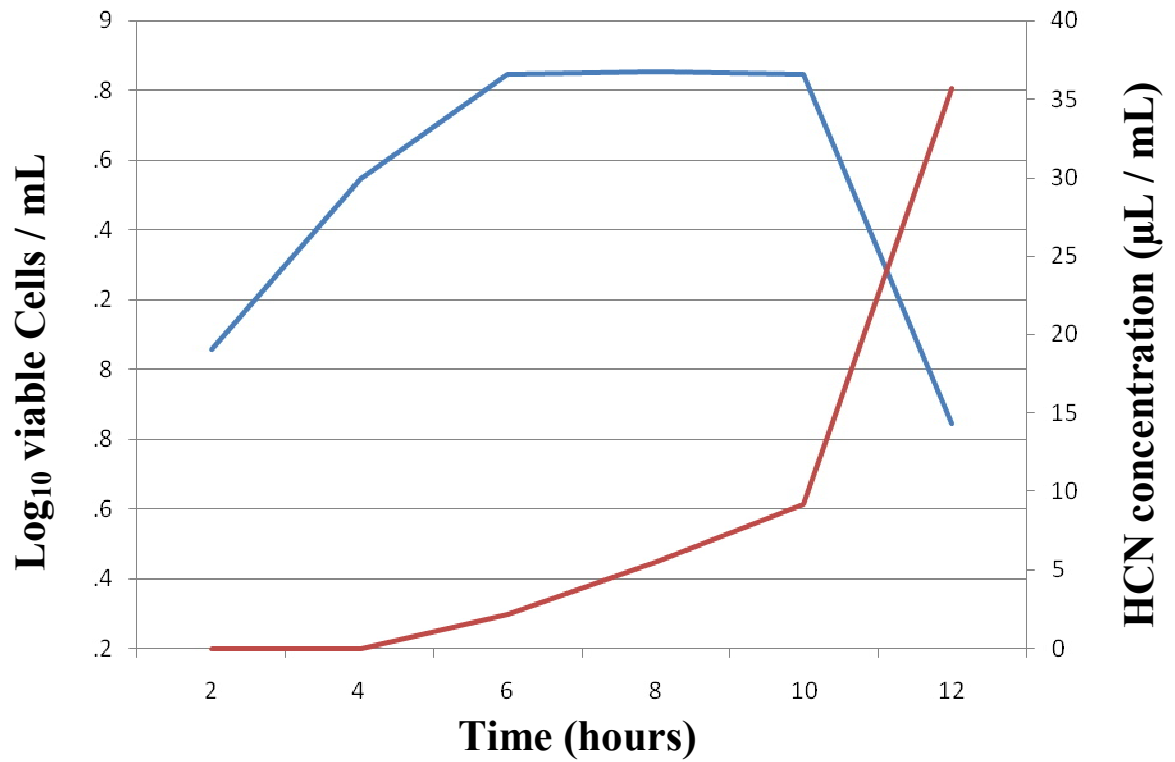




**Figure 3.2** Phenotypic characterization of the *Pseudomonas sp.* strain 15G2. A) exoprotease assay on skim milk agar medium (notice the clear zone around bacterial growth). B) Polysaccharides production on Pseudomonas agar. C) Fluorescence production on Pseudomonas agar plate under UV light. D) Activity against *Agrobacterium rhizogens* (notice the clear halo around the bacterial growth). E) Hydrogen cyanide production (notice the blue color of the detection paper). F) Siderophore production on the CAS plates (notice the orange halos around the bacterial growth)



**Figure 3.3** Epifluorescent microscopy image of soybean roots colonized by *Pseudomonas sp.* strain 15G2 tagged with green fluorescent protein (GFP). Soybean seeds were inoculated 7 days after germination with a 10 mL suspension of 15G2-gfp at  $10^9$  cfu/mL. Plants were grown in non-sterile sand and harvested 2 days after inoculation. A) Soybean roots after 2 day inoculation by *Escherichia coli* harboring GFP plasmid. B) Soybean roots colonized by 15G2-gfp strain.



**Figure 3.4** HCN production by *Pseudomonas sp.* strain 15G2 during bacterial growth.

The production of HCN occurs when cells enter the stationary phase. Symbols; —, cyanide concentration ( $\mu\text{M} / \text{mL}$ ); —, viable cells.

### 3.3.3 *Pseudomonas* sp. strain 15G2 mutants with loss of activity against *C. elegans*.

To help identify genes of the strain 15G2 that are responsible or contribute to the bacterial lethality toward *C. elegans*, we used the EZ::TN<sup>TM</sup> <R6Kγori/KAN-2> Transposome system to create the 15G2-Tn5 mutants. Approximately 2,700 15G2-mutants were generated and picked without regard to colony morphology or size. Individual mutants were screened for their activity against the *C. elegans* in a seven-day assay. Among all Tn5 mutants, 74 strains were significantly defective in killing the *C. elegans* (Table 3.3). Because the transposon inserts contained a kanamycin selectable marker and an *E. coli* origin of replication, we were able to rescue the plasmids and sequence the flanking regions of each transposon to identify the disrupted genes. Since no full genomic sequence was available for strain 15G2, we compared the DNA sequences of transposon flanking regions to all sequences available in the *Pseudomonas* database and determined the insertion sites according to their homologous sequences. The BLASTN searches revealed that 66 genes were highly homologous to *P. fluorescens* strain Pf-5 and two to *P. fluorescens* strain Pf0-1. Six genes had no homology to any of the available sequences in the *Pseudomonas* databases. We obtained strains Pf-5 and Pf0-1 and determined that neither of these strains is lethal to *C. elegans*. The genes whose disruption leads to loss of activity against *C. elegans* included genes encoding different cellular metabolic enzymes, transporters, proteases, regulators, and others with no assigned function (Table 3.3).

Our previous data (*Chapter II*) demonstrated that HCN is widely produced among most of our nematode-lethal *Pseudomonas* isolates but is probably not the sole factor for nematode lethality. HCN produced by the strain 15G2 –Tn5 mutants were evaluated to

determine whether or not they produced HCN. Our results showed that many of these mutants were no longer HCN producers while others produced it at variable levels (Table 3.3). In order to eliminate the possibility that losing the activity against *C. elegans* was not due to changes in bacterial growth rates, we compared the growth rates of strain 15G2 and the 74 Tn5 mutants on MM, NGM, and LB media. Only those with growth rates comparable to the WT-15G2 on LB media (Figure 3.18) were chosen for further analysis (highlighted in Table 3.3). For those strains with similar growth rates, the HCN production was measured on LB media (Figure 3.15). 3.7 to 27 fold reductions in HCN levels were obtained in 15G2-Tn mutants (Figure 3.15). In addition, we measured the HCN production by WT strain 15G2 and 15G2-transposon mutants growing on three different media: LB, MM, and NGM media (Figure 3.19). The results showed that highest HCN production was obtained when bacteria were grown on LB media and the production was significantly reduced when bacteria were grown on either MM or NGM media (Figure 3.19). The data from the above experiments indicate that HCN contributes one of the major lethal factors toward *C. elegans*. Since we did not obtain any chromosomal Tn5 transposon insertions in the *hcnABC* locus of strain 15G2 genome, different mutations in the *hcnABC* locus were created. Descriptions of constructing and evaluating these mutants are discussed later in this Chapter.

**Table 3.3** *Pseudomonas* sp. strain 15G2 Tn5 mutants defective in *C. elegans* killing

Strain	Insertion site <sup>a</sup>	Function <sup>b</sup>	HCN <sup>c</sup>
10-F7	10836	CHASE domain/sensory box histidine kinase	1
18-F1	115342	NAD(P) transhydrogenase, beta subunit	3
9-F8	115342	NAD(P) transhydrogenase, beta subunit	1
<b>18-F6</b>	<b>115342</b>	<b>NAD(P) transhydrogenase, beta subunit</b>	<b>1</b>
B2	115342	NAD(P) transhydrogenase, beta subunit	1
<b>16-C8</b>	<b>1199450</b>	<b>general amino acid ABC transporter, periplasmic binding p...</b>	<b>1</b>
19-B4	1318082	conserved hypothetical protein	2
H5	1491541	3-oxoadipate:succinyl-CoA transferase, A subunit	2
13-F7	1636680	pyochelin synthetase F	3
16-E6	1707587	tricarboxylate transport protein TctA, putative	3
15-D1	1807309	sigma-54 dependent transcriptional regulator PhhR	1
14-G1	18432	lipid A biosynthesis lauroyl acyltransferase	1
19-C10	1976692	5'-nucleotidase, putative	2
1B7	2086210	dihydroorotate oxidase	0
12-D12	2116167	transcriptional regulator, LysR family	1
9-E3	2116167	transcriptional regulator, LysR family	1
12-D10	2127298	alcohol dehydrogenase, zinc-containing	1
<b>9-A9</b>	<b>2145126</b>	<b>cytochrome c oxidase, cbb3-type, subunit I</b>	<b>3</b>
16-H3	2176375	LexA repressor	2
13-C7	2196858	heavy metal sensor histidine kinase	3
18-E8	2205949	TIM-barrel protein, yjbN family	2
13-C8	2227912	baseplate assembly protein V	3
17-E2	2541174	Predicted permease family	1
15-A1	2703333	ankyrin repeat protein	1
1C6	2726070	transcriptional regulator, LysR family	2
14-G11	3348465	toxin secretion ABC transporter protein, HlyB family	3
14-H3	3627264	conserved hypothetical protein	2
9-G11	3942322	1514 bp at 5' side: hypothetical protein	1
13-E4	4123128	membrane protein, putative	1
15-F4	423691	2,3-bisphosphoglycerate-independent phosphoglycerate mutase	1
<b>9-H12</b>	<b>464402</b>	<b>histidine transporter, periplasmic histidine-binding protein</b>	<b>2</b>
11-A2	4672855	membrane protein, putative	1
10-D11	5001116	competence protein comEA	2
<b>11-D4</b>	<b>5076374</b>	<b>xanthine/uracil permease family protein</b>	<b>3</b>
13-A2	5076374	xanthine/uracil permease family protein	2
17-E9	5121104	sensory box histidine kinase/response regulator	2
5F1	5135197	cobyric acid synthase CobQ	2
3H3A	5163540	D-lactate dehydrogenase	2
<b>G3</b>	<b>5163540</b>	<b>D-lactate dehydrogenase</b>	<b>0</b>
<b>A1</b>	<b>5163540</b>	<b>D-lactate dehydrogenase</b>	<b>1</b>

<b>E1</b>	<b>5163540</b>	<b>D-lactate dehydrogenase</b>	<b>1</b>
3H3B	5163540	D-lactate dehydrogenase	2
C2	5243341	hypothetical protein	2
<b>C3</b>	<b>5243341</b>	<b>hypothetical protein</b>	<b>2</b>
3C4	5603087	oxidoreductase, molybdopterin-binding	2
14-B6	5613439	glycerol-3-phosphate dehydrogenase, FAD-dependent	1
H3	5615342	amino acid ABC transporter, periplasmic amino acid-bindin...	2
<b>13-A10</b>	<b>5647927</b>	<b>1472 bp at 5' side: amino acid permease</b>	<b>2</b>
E5	5647927	1472 bp at 5' side: amino acid permease	1
G2	5672609	ATP-dependent protease La	2
G5	5672609	ATP-dependent protease La	1
C4	5680583	phosphoenolpyruvate-protein phosphotransferase	3
<b>18-E3</b>	<b>5779957</b>	<b>amino acid ABC transporter, permease protein VC1862</b>	<b>1</b>
20-A9	6032417	sugar fermentation stimulation protein	3
17-C7	6147586	heme transport protein, putative	1
12-E11	6245923	ethanolamine ammonia-lyase, large subunit	2
<b>11-E11</b>	<b>6482293</b>	<b>acyl-CoA dehydrogenase family protein</b>	<b>3</b>
2-H4	6618242	hemolysin III	2
3B10	6618242	hemolysin III	2
<b>2-D7</b>	<b>6618242</b>	<b>hemolysin III</b>	<b>1</b>
1E7	6645826	twitching motility protein	1
A5	6663898	Peptidase M16 inactive domain family	2
<b>18-A1</b>	<b>6723346</b>	<b>amino acid ABC transporter, periplasmic amino acid-bindin...</b>	<b>1</b>
14-G7	6923022	Rhs-family protein	3
1E6	6996185	sensory box-containing diguanylate cyclase, putative	1
16-F10	7067935	phytanoyl-CoA dioxygenase, PhyH family	1
9-A6	717476	membrane protein, putative	2
9-A7	717476	membrane protein, putative	3
17-C5	none	sensor histidine kinase/response regulator ChpA, putative	2
13-F5	none	AF127374	2
A3	none	AF396083.	2
15-F2	none	ring-hydroxylating dioxygenase, ferredoxin reductase subunit	3
D3	none	probable major facilitator superfamily (MFS) transporter(Class 3	2
Pioneer	none	heat shock protein HslVU, ATPase subunit HslU	0

a,b the insertion sites and function corresponds to the chromosomal location in *P. fluorescens* strain Pf-5

c, qualitative measurements of the HCN production scored 0-3 according to the production with three representing the highest level.

### 3.3.4 Indirect exposure of *C. elegans* to strain 15G2 cyanide

HCN produced by *Pseudomonas aeruginosa* PAO1 was demonstrated to be a primary virulence factor responsible for killing *C. elegans*. Exposure of *C. elegans* to 1 $\mu$ mol of cyanide gas was enough to kill the nematodes in 10 hours (Gallagher and Manoil, 2001). Since *Pseudomonas sp.* strain 15G2 produces HCN at comparable levels to kill *C. elegans*, we questioned whether the HCN produced by 15G2 alone is enough to kill the nematode or if there are other factors that may contribute to the bacterial lethality. In order to address this question, we studied the effect of strain 15G2 and three of the 15G2-Tn5 mutants (A1, G3, and 18-E3) on *C. elegans*. In these assays we permit the *C. elegans* and bacterial strains to share the same air in a sealed compartment and determined the nematode mortality over time. As negative controls, LB plates without bacterial inoculation or inoculated with *E. coli* were used. The nematode was deemed dead if it failed to move or respond to tapping the plate against the microscope stage. The number of dead nematodes was recorded every two hours. The results showed that WT-15G2 strain was able to cause 100% lethality after 6 hours of shared-air incubation, and neither *E. coli* nor 15G2 mutants were able to kill the nematode by the end of our experimental time points (Figure 3.5). The inability of *E. coli* and 15G2-Tn5 mutants to kill the nematode suggested that the previous killing was due to the HCN produced, which in turn may reduce the oxygen levels in the sealed plates. Additionally, to determine if the strain 15G2 also uses HCN as a primary mechanism to kill plant-parasitic nematodes, a similar experiment to those described above was performed using second-stage juveniles (J2) of *M. incognita* (RKN) instead of the *C. elegans*. The results were similar to those obtained above, except that the RKN-J2s were more resistant and



took a longer period of time to die (Figure 3.5). From these data, we concluded that HCN represents a primary toxic factor produced by the 15G2 strain and is responsible for killing both free-living and plant-parasitic nematodes *in vitro*.

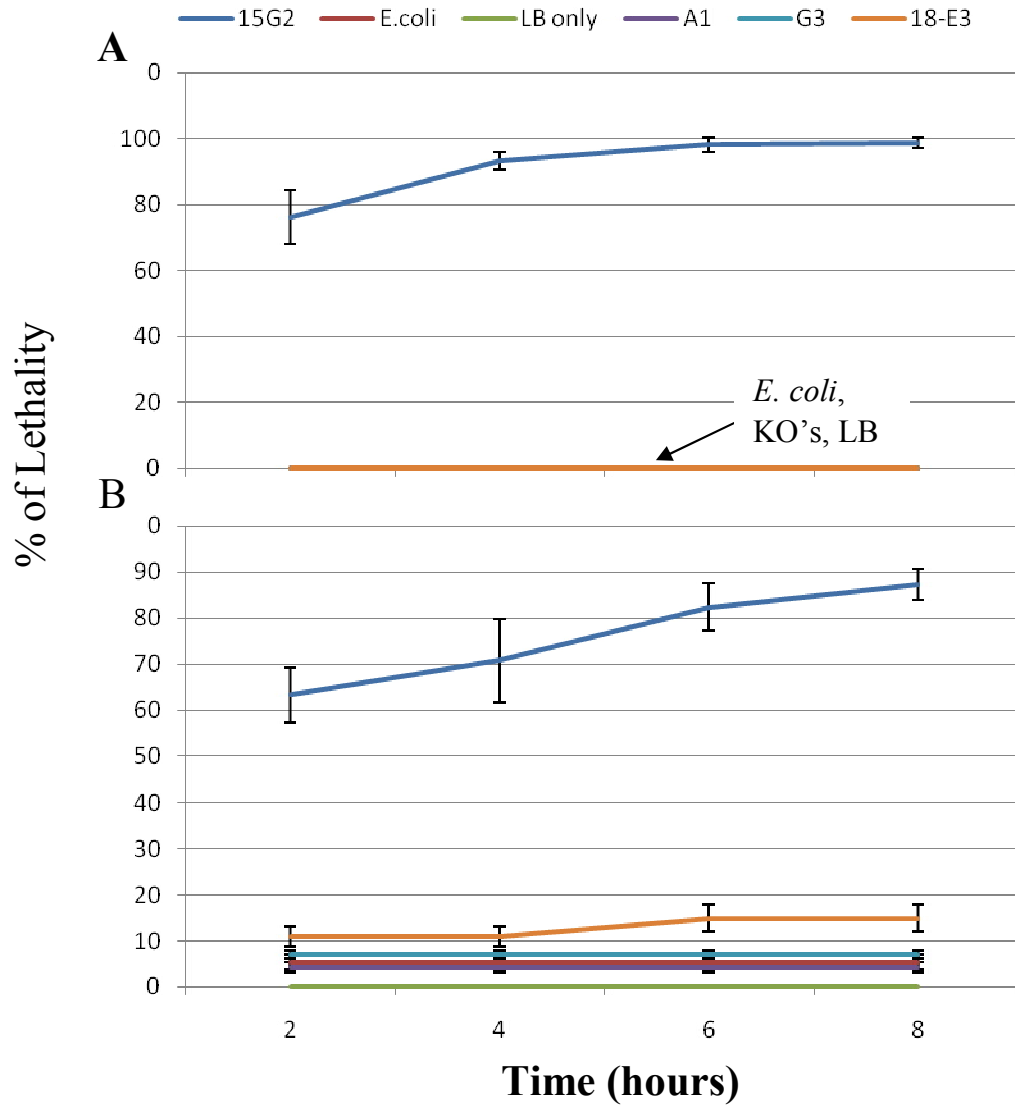
### 3.3.5 Amino acid transporters are involved in hydrogen cyanide production

Several studies have shown that some amino acids such as glycine, methionine, leucine, alanine, phenylalanine, tyrosine and serine can serve as precursors for hydrogen cyanide production (Castric, 1977; Johnson and Kan, 2002). Three of 15G2-Tn5 mutants (*16-C8*, *9-H12*, and *18-A1*) carried an insertion in genes encoding for a general amino acid ABC transporter-periplasmic binding protein, a histidine transporter-periplasmic histidine-binding protein, and amino acid ABC transporter-periplasmic amino acid-binding protein (Table 3.3). To determine if these genes are involved in the HCN production in strain 15G2, the ability of these mutants to grow on different amino acids and produce HCN were investigated. The effect of the presence of various amino acids as C and sole N sources in M9 medium on the growth of strain 15G2, *16-C8*, *9-H12*, and *18-A1* mutants were tested (Figure 3.6). Various amino acids were supplied at a 10 mM concentration and their growth rates (Figure 3.6) and HCN production (Figure 3.7) were measured. The results showed that nine amino acids (alanine, arginine, asparagine, aspartate, glutamate, glutamine, histidine, proline, and serine) can readily be used as a C and/or N source by strain 15G2 (Figure 3.7). However, *16-C8*, *9-H12*, and *18-A1* mutants had variable growth rates on M9 media supplemented with amino acids (Figure 3.6). In addition, our results showed the inability of *9-H12* mutant to utilize amino acid histidine, confirming the BLASTN results that this gene encodes for a periplasmic

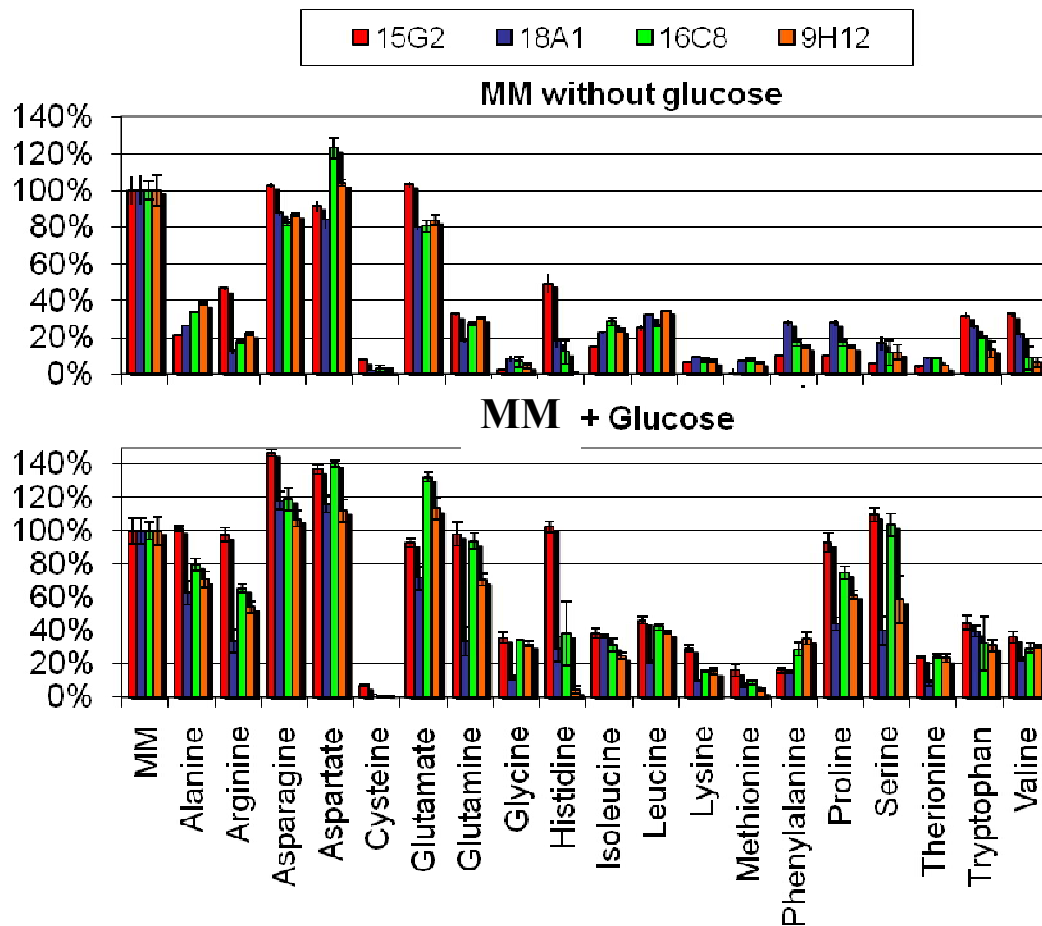
histidine transporter. However, *16-C8* and *18-A1* mutants do function in the transportation of general amino acids (Figure 3.6).

Next, we examined the ability of these strains to use different amino acids as a precursor for HCN production. Strains were grown on M9 agar media supplemented with various amino acids and hydrogen cyanide detection paper was placed on the inside of the plate lid. The results showed that both strain 15G2 and *18-A1* mutant were able to strongly produce HCN when growing on all media except those supplemented with cysteine, methionine, phenylalanine and threonine (Figure 3.7). In contrast, both *16-C8* and *9-H12* mutants did not produce or produced very small amounts of HCN on all media tested (Figure 3.7). The HCN production by WT strain 15G2 growing on MM supplemented with different amino acids (1  $\mu$ M) was quantified using a cyanide electrode (Figure 3.20). The highest HCN production was obtained when bacteria were grown on MM supplemented with either arginine or glycine (Figure 3.20).

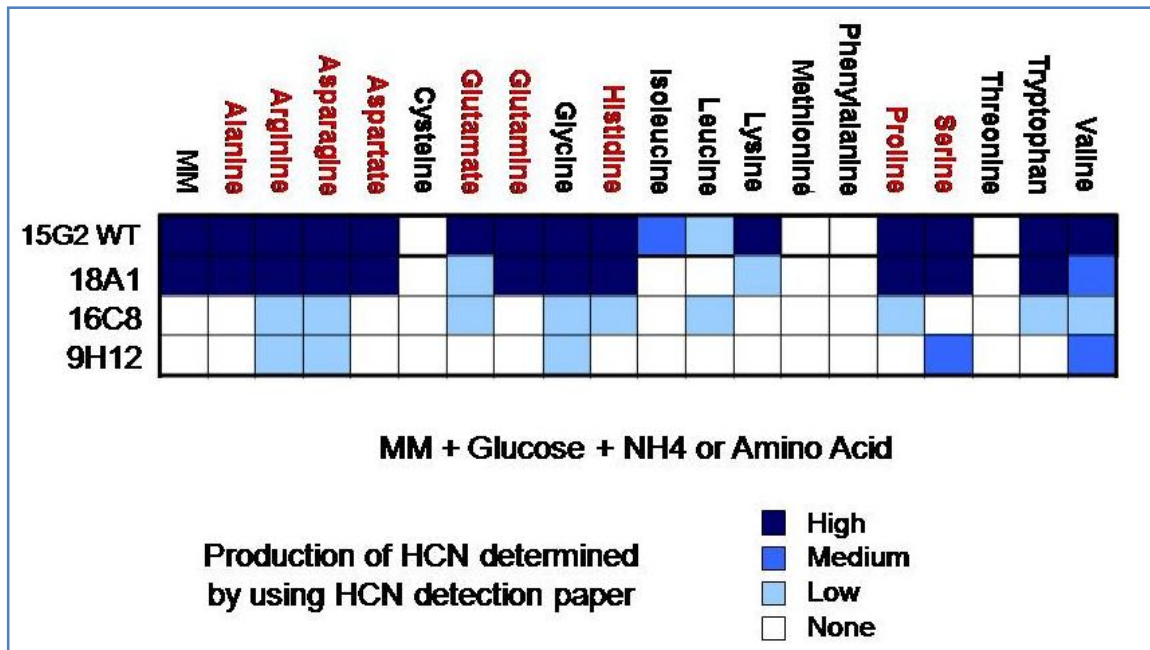
To verify that *16-C8*, *9-H12*, and *18-A1* genes are involved in some way with hydrogen cyanide production and not just a result of the polar effects of the transposon insertions, *16-C8*, *9-H12*, and *18-A1* mutants were complemented with the corresponding wild-type genes carried on broad-host-range plasmid CGT 4632A. The hydrogen cyanide quantification analysis for these complemented strains revealed that the complemented *9-H12* strain was able to produce approximately 91% HCN compared to strain 15G2 (Figure 3.8). However, the complemented strain *18-A1* and *16-C8* produced 77% and 61% HCN, respectively, compared to strain 15G2 (Figure 3.8). Protein subcellular localization prediction for *16-C8*, *9-H12*, and *18-A1* amino acid sequences showed that these proteins possess cytoplasmic membrane targeting signals.



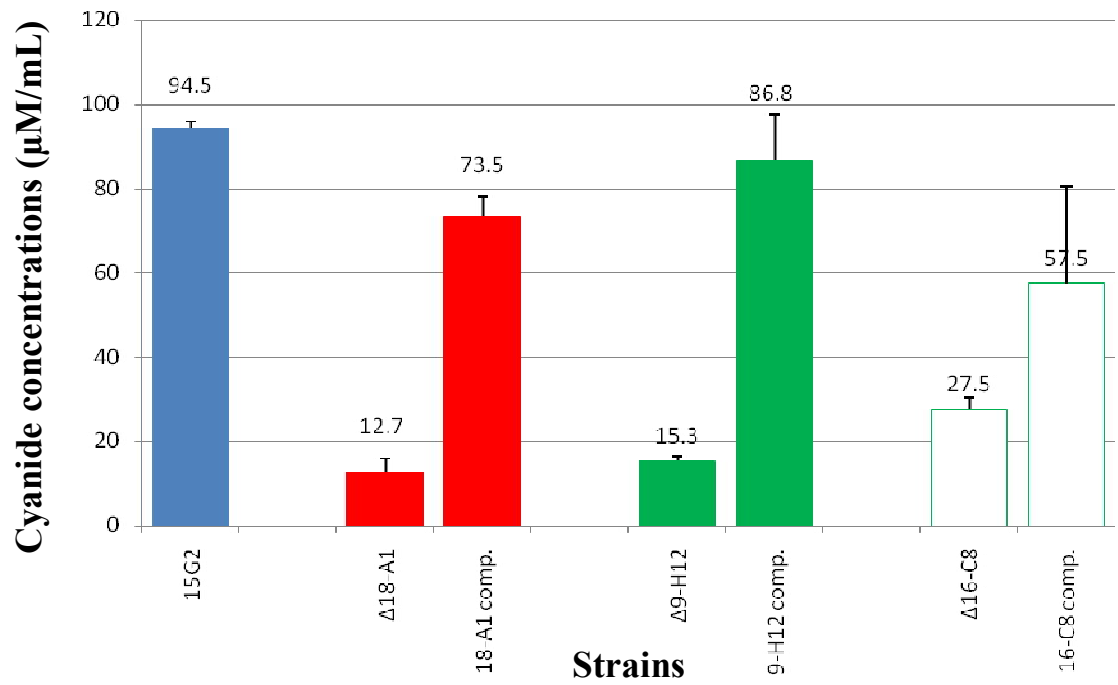
**Figure 3.5** Indirect exposures of *C. elegans* and root-knot nematodes to *Pseudomonas* *sp.* strain 15G2 and its knock-outs. Nematode J2's were allowed to share the same air with bacterial strains in a sealed compartment and the percentage of killing was determined over time for *C. elegans* (A) and Root-knot nematodes (*M. incognita*) (B).



**Figure 3.6** Growth rates of wild type *Pseudomonas sp.* strain 15G2 and 15G 2-Tn5 mutants (9-H12, 18-A1, and 16C18) on minimal media supplemented with different amino acids. A) Growth rates for bacteria growing on MM without glucose. B) Growth rates for bacteria growing on MM plus glucose. MM represents minimal medium plus glucose and NH<sub>4</sub>Cl.



**Figure 3.7** Hydrogen cyanide production of *Pseudomonas sp.* strain 15G2 and 15G 2-Tn5 mutants (*9-H12*, *18-A1*, and *16C18*) growing on minimal media supplemented with different amino acids.



**Figure 3.8** HCN production by *Pseudomonas* sp. strain 15G2 and 15G2-Tn5 mutants (*9-H12*, *18-A1*, and *16C18*) and complemented mutants growing on LB media.

### 3.3.6 Identification and characterization of the *Pseudomonas* sp. strain 15G2 hydrogen cyanide gene cluster (*hcnABC*)

To determine the *hcnABC* nucleotide sequences of *Pseudomonas* sp. strain 15G2, we obtained the *hcnABC* sequences of *P. fluorescens* strain Pf-5 and strain Pf0-1, *P. entomophila* L48, *P. aeruginosa* PAO1, and *P. aeruginosa* PA14 from the *Pseudomonas* Genome database. Based on sequence similarities, we designed two sets of primers to partially amplify *hcnB* and *hcnC* genes. The sequence analysis of the resulting fragments of strain 15G2 showed 89 and 92% similarity with *hcnB* and *hcnC* genes of *P. fluorescens* strain Pf-5, respectively. Then we used BWZ and CIB primers to obtain the sequence between *hcnB* and *hcnC* partial sequences. In order to get the full sequence of *hcnABC* of strain 15G2-, we designed primers at flanking sequences to amplify the entire operon according to the strain Pf-5 sequences. Unfortunately, no amplification products were obtained even when using LongAmp™ *Taq* PCR. We used a chromosome walking approach to get the full *hcnABC* sequences plus 1 to 2 kb of the 5' and 3' flanking sequences. Using the self-formed adaptor PCR (SEFA-PCR) method (Wang et al., 2007), we obtained the full sequence of *hcnABC* (2.97 Kb), 0.9 kb of the 5' flanking sequences, and 2 kb of the 3' flanking sequences (Figure 3.9). The nucleotide sequence of the *hcnABC* gene cluster of strain 15G2 revealed three open reading frames highly homologous to the *hcnA*, *hcnB*, and *hcnC* of *P. fluorescens* Pf-5 (Figure 3.10). A number of motifs have been identified and suggested to be important for *hcnABC* regulation and production (Laville et al., 1998; Pessi and Haas, 2000; Ryall et al., 2008). We analyzed the 15G2 *hcnABC* sequence and found several motifs and structures that are conserved among the *Pseudomonas* spp. *hcnABC*. For example, the ANR box, transcription start

site (Figure 3.10), Fe-S binding site in *hcnA* gene and the 11 amino acid residues that represent ADP-binding motif in *hcnB* and *hcnC* were present.

The alignment of the amino acid sequences deduced from the strain 15G2 *hcnABC* with that of *P. fluorescens* strain Pf-5 revealed 94% similarity between the two strains (Figure 3.11). In contrast, the nucleotide sequences flanking the 5' and 3' ends of the *hcnABC* of strain 15G2 on a stretch of 1000 bp share 53 and 63%, respectively (Figure 3.12 & 3.13), with the corresponding regions of the strain Pf-5.

### **3.3.7 Construction and properties of the *Pseudomonas* sp. strain 15G2 *hcn* mutants**

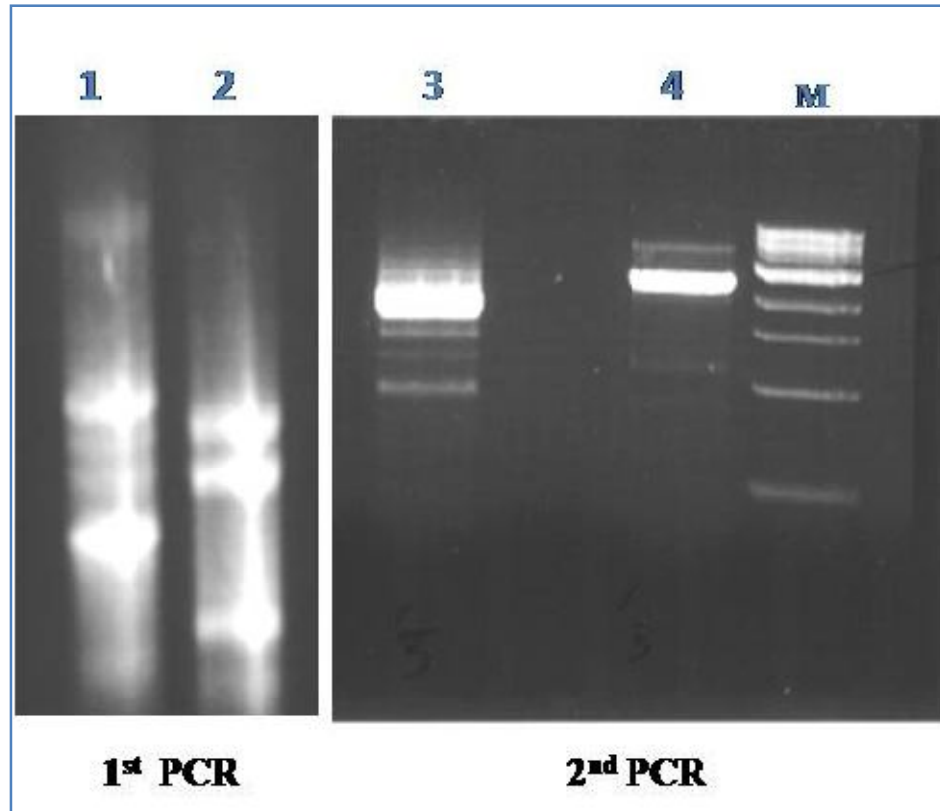
In order to determine if the *hcnABC* gene cluster of 15G2 strain is responsible for its HCN production, four different chromosomal deletions in this locus were generated. First, we disrupted the entire *hcnABC* locus by insertion of the kanamycin resistance gene, which was introduced into the 15G2 chromosome by the use of suicide vector pCGT 9033 and double crossover as described in Materials and Methods. Second, to determine whether these genes form an operon and together are responsible for the HCN production or only one gene does this function, we created similarly single chromosomal deletions,  $\Delta hcnA$ ,  $\Delta hcnB$ , and  $\Delta hcnC$ , by using the suicide vectors pCGT 9036, pCGT 9040, and pCGT 9043, respectively. The chromosomal deletions were confirmed using PCR and sequencing approaches. Southern blot hybridizations were carried out to determine the copy number of *hcnABC* in strain 15G2 and to confirm the construction of 15G2- $\Delta hcnABC$  mutant. The analysis of the complete genome sequence of the *Pseudomonas* sp. strain Pf-5 revealed that there is only one copy of the *hcnABC* genes;



thus, we used this strain as a control. Genomic DNA samples from WT strain 15G2, 15G2- $\Delta hcnABC$  mutant, and strain Pf-5 were digested with *EcoRI* and *KpnI* enzyme and resolved on an agarose gel that was blotted and hybridized with a labeled 650 bp fragment of the *hcnB* gene. The results confirmed the presence of one copy of the *hcnABC* gene cluster in strain 15G2 compared to the Pf-5 and revealed these genes were absent from the 15G2- $\Delta hcnABC$  mutant (Figure 3.16). The growth rates of these mutants were similar to that of the WT (Figure 3.15). To determine the HCN-phenotypes of these mutants, we evaluated the HCN production qualitatively and quantitatively. Our results from the qualitative assay showed that all mutants had HCN- negative phenotypes on the assay plates. In addition, the qualitative measurements of HCN production by these mutants was compared to that of the WT stain 15G2 using a cyanide-selective electrode (Figure 3.14). The  $\Delta hcnABC$  mutant produced only about 5  $\mu\text{M}$  HCN, which is considered to be a negative-producer strain according to Laville et al. (1998). Similarly,  $\Delta hcnA$ ,  $\Delta hcnB$ , and  $\Delta hcnC$  mutants were strongly impaired in their ability to produce HCN (Figure 3.14). These results indicate that *hcnABC* is likely responsible for high levels of HCN production in strain 15G2. However, a low level of cyanide is produced in these knockouts suggesting that another mechanism for cyanide production may also be present (Figure 3.14). To determine if any of these genes could lead to HCN production by themselves, *hcnB* and *hcnC* were cloned in the broad-host range-plasmid pCGT 4632A and transformed to *E. coli*. This bacterium does not produce HCN and does not possess the HCN detoxification enzymes. Our results showed that transforming the *hcnB* or *hcnC* to *E. coli* leads to cell death indicating that HCN can be produced by either enzyme alone (data not shown). Similar results were obtained when expressing the

*hcnABC* operon from *P. fluorescens* CH01 behind the T7 promoter and transforming it to *E. coli* cells (Laville et al., 1998).

Indirect exposure of *C. elegans* L2s to the *hcnABC* mutants did not result in killing the nematode as predicted, indicating that HCN is a major factor in killing the nematode without direct contact. In contrast, direct exposure of the *C. elegans* L2s to *hcnABC* mutants in the bioassay medium resulted in killing the nematode. However, they killed the nematode over a longer time period (5 days) compared to WT strain 15G2 (one day). These results may imply that low levels of HCN production or other substances can contribute to *C. elegans* lethality in this strain.



**Figure 3.9** Chromosome walking of *Pseudomonas* sp. strain 15G2 genomic DNA. Lane 1 and Lane 4 are the products of the first and second round of SEFA PCR for the 3' flanking region, respectively. Lane 2 and lane 3 are the products of the first and second round of SEFA PCR for the 5' flanking region, respectively. Lane M is 1K ladder marker; from bottom to top, 0.5, 1, 1.5, 2, 3, 4, 5, 6, 8 and 10 kb.

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1  AAGGCACTGC TTTGAGGGAC ACCGTCCTTT TGCATGGGCC AGATCAATAT TTCTGTGAGA CAAAAACAC
71  AGATTTGCCC GCCTCGGTT CTGACAGCCA CTTCGGAAAC GCGGTGTGAC CGCGTCTTG CCCCAGGACT
      M R Q I D R N
141  CCAGGCGGCA GGATGCCGGI AACCCCACTC ACTTCTCAAG GATGAACCGC AATGGGTCAG ATAGACCGCA
      F D I Q P L Q H A D M T I S L N G Q P V T A A
211  AITTTGATAT TCAGCCGCTG CAGCATGCGG ACATGACCAT CAGTCTCAAC GGCCAGCCGG TCACGTGCCGC
      L G E T V L S V I Q A T G L R Q V A R N D H G
281  CCTGGGTGAA ACAGTCTTCA GCGTATCCA GGCCACCCGG CTGCGCCAGG TGGCGCGCAA TGATCAGCGA
      Q L V G A Y C G M G V C H C C L V Q I D G R H K
351  CAGTTGGTGG GTGCCTACTG CCGCATGGGC GTGTGCCATT GCIGCCTGGT GCAGATCGAC GGCCGCCACA
      R R A C Q T L V K P G M Q V Q T L S N R I S E
421  AGCGCCGCTG CTGCCAGACC CTAGTCAAGC CGGGCATGCA GGTGCAGACC CTGAGCAACC GCATCAGCGA
      T E P T V
      M S L N P V I V G G G P A G M A A A I
491  GACGGAGCCC ACGGTATGAS CTGAACCCGG GTGATGTGG GCGGAGGACC GCGGATATG GCGCGGCTA
      E L A K H G V R S T L I E E A S R L G G V V F
561  CTGACCTGGC CAASCACGGC GTGCGCAGCA CCGTATCGA AGAAGCCGAG GCGGTGGGTC GCGCGGCTT
      R G P L R D G V Q L D Y L G P R Y C E T L A K
631  GCGCGGCGCG GTGCGGATG GCGTCAACT GATTAACCTG GCGCGCGGCT ACTGCGAGAC GCGTGCAGG
      L H G E F S D H E Q M I E V R L N H R V I G A E
701  CTGACCTGGC AATTTTCCGA TCAAGAGTAC ATGATCGAGG TGCCTCTCAA CACCGCGGTC ATGCGTGGC
      G L D S L M L L D A D E Q V Q Q V A Y G Q L L
771  ACGGCTTTGA CAGCTGATG CTGCTGGATG CCGCAAGCA GGTGCAGTAC GTGGCTATG GCGATTTT
      L A A G C H E R S V P F P G W T L P G V K L L
841  GTCGCGGCCC GCGTGCATG AAGCAGCGT ACCGTTTCCC GCGTGGAGC TCGCGGCGGT CAGTGTCTC
      G G L Q L Q I K S G V V K P Q E P V V I A G T
911  GGTGCTTC AACTGCAGT CAGAGCGGTC GTGTTAAG CCGAAGTCC CCGTGTGATC GCGTGCATC
      P L L P L V A C Q L H A S G V R V A G V Y E A
981  GCGGCTTGGT GGCATGGTG GCGTGCAGI TGCAGCCCTC GCGCGTGGCG GTGGCGGCGG TGTATGAGC
      S A L S K I A K Q S L A L L N K P Q L F L D G
1051  CAGTGTCTG AGCAAGATG CCGAGCAGG CCGTGGCCCTG CTGAACAAGC CCGAGTGTI TGTGAGCGC
      L S M L A Y L K L H S I P L R Y G W G V E A Q
1121  CTGAGTATGC TGGCTATGCT CAGCTTTCAC AGCATCCCCC TGGCTATGG CCGGGGCGTG GTGGAGGCG
      G Q D A L S V V T V A P Y S A D W Q P D M A K
1191  AGGGCGGCGA CCGCTGAGT GTGTCATGG TGGCCCTTIA TCGCGCGAC TGGCAGCGGC ACATGGCCAA
      A E R I A A Q T L A V G Y G F I P R T Q L S Q
1261  GCGCGAGCGC ATCGCCGCGC AGACCTGGC GGTGGGTTAT GCGTTCATC CCGCGACCCA ACTGAGCGAG
      Q M G L D H G F S D D G Y L R A N A N A W Q Q S
1331  CAGATGGCCC TGGATCAGG CTTCAGGAT GACCGGCTATC TGGGGGCAA CCGCAACCGC TGGAGCAGA
      S E P H I H L A G D M G G I R G G E A A M L T
1401  GCGAGGAGCC GCATATCAT CCGCGCGGCG ACATGGGTCG GATTCGCGT GGTGAAGCGG CCGTGTCCAC
      G R I A A L S I L M Q R D V L S N E A A L Q R
1471  GCGCGGATC GCGCGGCTGT GATCTTCAT CCGAGCGGAT GGTGTGAGCA AGGAGGCGGC CTGTGAACGG
      R Q G Y E R K L A S I L R F R G A V D R Y T A R
1541  GCGCAGGCGT ACAGCGCAA ACTGGCCCTG ATCTGCGCT TTGGTGGGCG GTTGGAGCGC TATAGCGAC
      G T G Q I D L P K G D T V I C R C E H I T R N
1611  GTGGAGCGG SCAPATAGA CTGCCAAGG CCGACAGCGT GATTTGCGC TGTGAACACA CCACTGCAA
      D I E R A L S Q G V Q D M A S L K M R T R V S
1681  GATATGAGG TGGCCCTTGA GCGAGGCGGT GCAGGACATG GCGAGCGTGA AAATGGCCAC CCGGGTGA
      M G D C Q G R M C V G Y C S D R L R Q A T G R K
1751  ATGGCGACTI CCGAGGCGCG GATGTCCTC GCTATTGCA GCGACCGGCT GCGCCAGGCG ACCGAGCGA
      D V G W I R P R F P L D P I P F S A F A A P D
1821  AGGATGCGGG CTGGATTGC CCGCCCTCC GGTGGAGCC GATTCGCTI TCGCGCTTG CAGCCCGACA
      M S K Y D V V I A G G G V I G A S
      Q E V P G H E
1891  CCGGAGGTC CCGGAGTGT AGTATATAT AGAGCTGT GATTCGCTI GCGCGCTG TGGCGGCTI
      C A Y Q L S K R K D L R V A L I D A K R P G N
1961  GCGCGGCGCG GCGCGGCGCG GCGCGGCGCG GCGCGGCGCG GCGCGGCGCG GCGCGGCGCG GCGCGGCGCG
      A S R A E A G G L W A T G F S V G L G C G V T F
2031  GCGCGGCGCG GCGCGGCGCG GCGCGGCGCG GCGCGGCGCG GCGCGGCGCG GCGCGGCGCG GCGCGGCGCG
      F R M M S A N R K R E A Q G S A V V V D S S T
2101  GCGCGGCGCG GCGCGGCGCG GCGCGGCGCG GCGCGGCGCG GCGCGGCGCG GCGCGGCGCG GCGCGGCGCG
      P H I L P Q S F F D F A L Q S N E L Y P R L H
2171  GCGCGGCGCG GCGCGGCGCG GCGCGGCGCG GCGCGGCGCG GCGCGGCGCG GCGCGGCGCG GCGCGGCGCG
      R E L I G L H G M D F K F E Q T G L K F V I Y D
2241  GCGCGGCGCG GCGCGGCGCG GCGCGGCGCG GCGCGGCGCG GCGCGGCGCG GCGCGGCGCG GCGCGGCGCG
      D E D R L Y A E H I V G C I P H L S D Q V R W
2311  GCGCGGCGCG GCGCGGCGCG GCGCGGCGCG GCGCGGCGCG GCGCGGCGCG GCGCGGCGCG GCGCGGCGCG
      L D Q A A L R A A E P K V S H E A Q G A L E F
2381  GCGCGGCGCG GCGCGGCGCG GCGCGGCGCG GCGCGGCGCG GCGCGGCGCG GCGCGGCGCG GCGCGGCGCG
      L C D H Q V N P F R L T D A Y T E G A R Q N G V

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2451 CTGTGCSACC ACCAGSTTAA CCCCTTGGC TTGACTGATG CTTACACCGA AGGCCACGG CAGAACGGC
  • D V Y F N I N V I G V L H O G N R V S G V O T •
2521 TGGATGTGTA CTTCAACACC AACGTACCG GGGTCTGCA CCAGGCCAAC CCGGTGAGT GGTACAAAC
  • A E A G L F H C S T L I N A A G A W A A E I S •
2591 CCGCGAGGCC GGGCTGTTC ATTGCTGGC CDTGATCAAT GCGGCCGTT CTTGGGCCG CAGGCTGAG
  L Q A T G V E I P V K P V K G Q I L L T E R M P •
2661 CTTGAGGCLA CTTGGGTTCA GATTCGGT AACCCGGTGA AGGGCLAA CTTCTTACC CAGGCTAGT
  • K L L N G C L T I S D C Y N A Q K D N G E I L •
2731 CCAAGCTGCT CAATGGTTC CTGACCACCA GCGACTGTA CATGGCCGAG AAGGACACG CCGAGATCT
  • I G S T T E D K G F D V S T T Y P E I N G L V •
2801 GATTCGGTAC ACCCGCGAG CCGGGCTTT GAGCTGAGC ACCACTTTC CCGAATCAC CCGCTGCTG
  Q G A V R C I F E L A Q V N L K R C W A G L R P •
2871 CACCGCCAC TCCCTTCAT TCCGATTC CCGACCTCA ACCCAACCG CTTCTCCCG CCGCTCCCT
  • G S P D E L P I L G P M D G V E G Y L N A C G •
2941 CCGGCTCCG CAGACGATG CCGATTCTT GCGCGATGA TGGGGTCGAG GGTACTCTCA CCGCTCCCG
  • H F R I G I L I S A I I G V L L D K L V N E E •
3011 ACACTTCGCT ACCGGCAACC TACCTCGGC GATCACCGG CTACTGCTGG ACAAACTGGT GAACGACGA
  A L P L D I T E F L A R R F A T A P V K K Q P E •
3081 GCGCTGGCGC TGGACATCAC TCGTTCCTG GCGCGCGTT TCGCCATGC CCGGGTCAAG AAACAGCCG
  • P A * •
3151 AACGGGCTG ATAGATCCC

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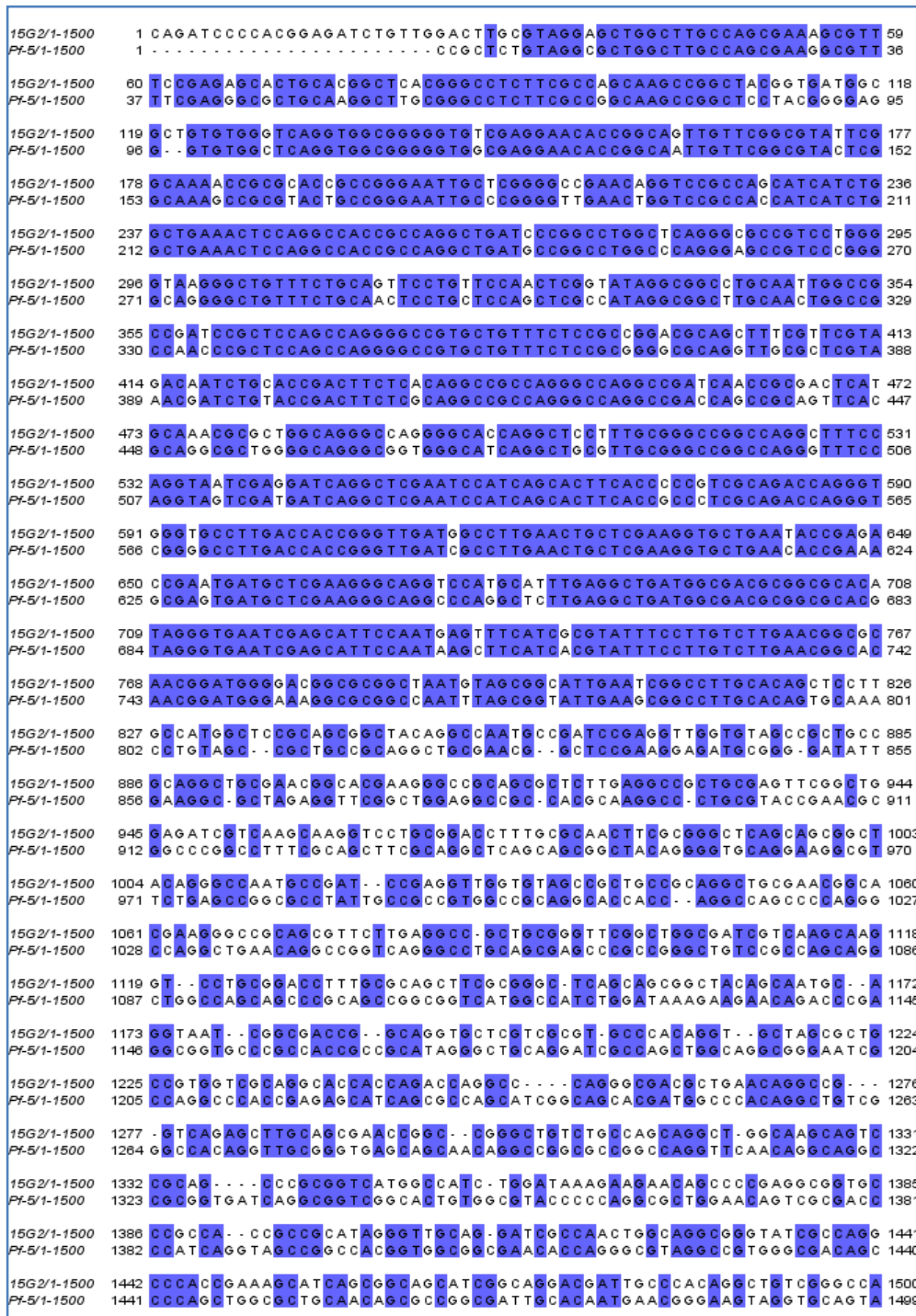
**Figure 3.10** Nucleotide sequence of the *hcnABC* operon of *Pseudomonas sp.* strain 15G2 and deduced amino acid sequence of its protein product. The color shaded nucleotides represent: yellow, the *hcnA* open reading frame (ORF); green, *hcnB*-ORF; pink, *hcnC*-ORF; gray, ANR-box; and red, transcription start site. The boxed amino acid residues represent the predicted transmembrane segments according to ConPred II transmembrane prediction server (<http://bioinfo.si.hirosaki-u.ac.jp/~ConPred2/>).

15G2- <i>hcnABC/1-991</i>	1	MRQIDRNFDIQPLQHADMTISLNGQPVTAAALGETVLSVIQATGLRQVARNDHGQLV	56
<i>Pf-5-hcnABC/1-991</i>	1	MRQIDRNFDIQPLQHADMTISLNGQPVTAAALGETVLSVIQATGLRQVARNDHGQLV	56
15G2- <i>hcnABC/1-991</i>	57	GAYCGMGVCHCCLVQIDGRHKRRACQTLVKPGMQVQTLNRISETTEPTVMSLNPVI	112
<i>Pf-5-hcnABC/1-991</i>	57	GAYCGMGVCHCCLVQIDGRHKRRACQTLVKPGMQVQTLNRISETTEPTVMSLNPVI	112
15G2- <i>hcnABC/1-991</i>	113	VGGGPAGMAAAIELAKHGVRSTLIEEASRLGGVVF RGPLRDGVQLDYLGP RYCE TL	168
<i>Pf-5-hcnABC/1-991</i>	113	VGGGPAGMAAAIELAEHGVRSTLIEEASRLGGVVYRGPLRDGVQLDYLGP RYCE ML	168
15G2- <i>hcnABC/1-991</i>	169	AKLHG E F SDHEQMI EVRLNHRV I GA E GLD S L M L L D A D E Q V Q V V A Y G Q L L L A A G C H E	224
<i>Pf-5-hcnABC/1-991</i>	169	AKLHG D F A D H E Q M I D V R L N S R V V G A E G T Q S L V L L D G E E Q V Q V V S Y E Q L I L A A G C H E	224
15G2- <i>hcnABC/1-991</i>	225	RSVPFPGWTLPGVKLLGGLQLQIKSGVVKPGSPVVIAGTGPLLPLVACQLHASGVR	280
<i>Pf-5-hcnABC/1-991</i>	225	RSVPFPGWTLPGVKLLGGLQLQIKSGVVKPGSPVVIAGTGPLLPLVACQLHASGVR	280
15G2- <i>hcnABC/1-991</i>	281	VAGVYEASALS KIAKQSLAL LNKPQLFDGLSMLAYLKLHS I PLRYGWGVVEAQQG	336
<i>Pf-5-hcnABC/1-991</i>	281	VAGVYEACALGKIAKQSLAMLNKPQLFDGLSMLAYLKLHG I ALRYGWGVVEAQQG	336
15G2- <i>hcnABC/1-991</i>	337	DALSVVTVAPYSA DWQPDMAKA ER IAAQTLAVGYGFI PRTQLSQQMGLD HGF SDDG	392
<i>Pf-5-hcnABC/1-991</i>	337	DALSVVTVAPYSS DWQPDMAKA QR IAAQTLAVGYGFI PRTQLSQQMGLE HNF SDDG	392
15G2- <i>hcnABC/1-991</i>	393	YLRANANAWQQSSEPHI HLAGDMGGIRGGEAAML TGRI AALSILMQRD VLSNEAAL	448
<i>Pf-5-hcnABC/1-991</i>	393	YLRASANAWQQSSEPHVHLAGDMGGIRGGEAAML SGRI AALSILMQRG VLSNEAAL	448
15G2- <i>hcnABC/1-991</i>	449	QRRQGYERKLASILRFRGAVDRYTARGTQQIDLPKGD TVICRCEHTTRNDIERALS	504
<i>Pf-5-hcnABC/1-991</i>	449	QRRQGYERKLASILRFRGAVDRYTARGAQQVELPKGD TVICRCEHTTRNDIERALS	504
15G2- <i>hcnABC/1-991</i>	505	QGVQDMASLKMRTRVSMGDCQGRMCGVYCSDRLRQATGRKDVGWIRPRFPLDPIPF	560
<i>Pf-5-hcnABC/1-991</i>	505	QGVQDMASLKMRTRVSMGDCQGRMCGVYCSDRLRQATGRKDVGWIRPRFPLDPIPF	560
15G2- <i>hcnABC/1-991</i>	561	SAFAAPDQEVPGHEMSKYDYDVVIAGGGVIGASCAYQLSKRKDLRVALIDAKRPGNA	616
<i>Pf-5-hcnABC/1-991</i>	561	SAFPPSDQEVSQHDMIKHYDVVIAGGGVIGASCAYQLSKRKDLKVALIDAKRPGNA	616
15G2- <i>hcnABC/1-991</i>	617	SRASAGGLWAI GESVGLGCGVIFFRMMSANRKREAGGS AVVVDSSTPHILPQSFFD	672
<i>Pf-5-hcnABC/1-991</i>	617	SRASAGGLWAI GESVGLGCGVIFFRMMSANRKREAGGS AVVVDSSTPHILPQSFFD	672
15G2- <i>hcnABC/1-991</i>	673	FALQSNELYPRLHREL IGLHGMDFKFEQTGLKFVIYD DEDRLYAEHIVGCIPHLS D	728
<i>Pf-5-hcnABC/1-991</i>	673	FALQSNELYPRLHRELMGLHNMDFKFEQTGLKFVIYDEEDRLYAEHIVGCIPHLS D	728
15G2- <i>hcnABC/1-991</i>	729	QVRWLDQAALRAAEPKVSHEAQQALEFLCDHQVNPFR L TDAYTEGARQNGVDVYFN	784
<i>Pf-5-hcnABC/1-991</i>	729	QVRWLDQAALRASEP NVSHEAQQALEFLCDHQVNPFR L TDAYTEGARQNGVDVYFN	784
15G2- <i>hcnABC/1-991</i>	785	TNVTGVLHQGNRVSGVQTAEAGLFRHCS TLINAAGAWAAELSLQATGVEIPVKPVKG	840
<i>Pf-5-hcnABC/1-991</i>	785	TNVTGVLHQGNRVSGVKT DVAGLFRCT TLINAAGAWAAELSLQATGIEIPVKPVKG	840
15G2- <i>hcnABC/1-991</i>	841	QILLTERMPKLLNGCLTTSDCYMAQKDNGEILIGSTTEDKGF DVSTTYPEINGLVQ	896
<i>Pf-5-hcnABC/1-991</i>	841	QILLTERMPKLLNGCLTTSDCYMAQKDNGEILIGSTTEDKGF DVSTTYPEINGLVQ	896
15G2- <i>hcnABC/1-991</i>	897	GAVRC IPELAQVNLKRCWAGLRPGSPDELPI LGPMDGVEGYLNACGHFR TGI L T S A	952
<i>Pf-5-hcnABC/1-991</i>	897	GAVRC VPELAHVNLKRCWAGLRPGSPDELPI LGPMDGVEGYLNACGHFR TGI L T S A	952
15G2- <i>hcnABC/1-991</i>	953	ITGVLLDKLVNDEALPLDITPFLARRFATA PVKKQPEPA	991
<i>Pf-5-hcnABC/1-991</i>	953	ITGVLLDKLVNDEALPLDITPFLARRFATT PVKKQPEPA	991

**Figure 3.11** Alignment of deduced amino acids sequences of *hcnABC* gene cluster for *Pseudomonas sp.* strain 15G2 and *P. fluorescens* strain Pf-5. The identity in amino acid sequence between bacterial strains is approximately 94%. The alignment was made by the ClustalW2 program (<http://www.ebi.ac.uk/Tools/clustalw2/index.html>). Identical residues are shaded blue.

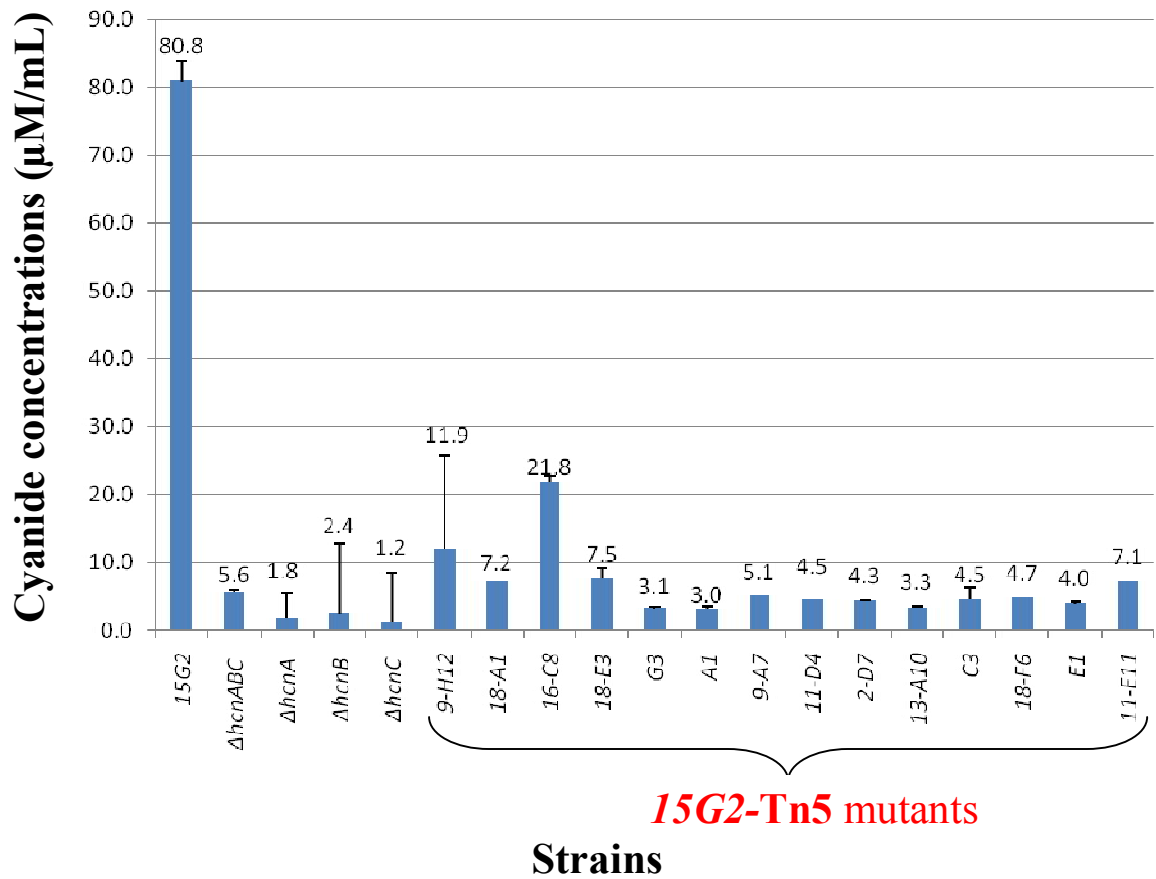
15G2/1-941	1	CAGGGGGCATT	TGCAAGAAACCAGGGGGGGATTCTAAATAAACTTTTCATCTGGCTT	61
Pf-5/1-941	1	-AGCAGCGTCAT	CAGCGGCGGCGTGCATGCCGGCGCCAGGTGGCTTCCGGCGCCCGCG	59
15G2/1-941	82	TCGTCCGGT	TGCACAGTCCGAGTCGGGAGGGTGAAGCTTGATATCATC-. . . .CGGCCAG	118
Pf-5/1-941	80	TGCCATGACC	CAGGTGGCCGGCCATGCTGCCGCCAGGGTTCCCAGGATGACTACCGCTAC	120
15G2/1-941	119	TTTTTCGCCAGGAGG	CATCACCATGAGTGCCTGACTCGGGTCGGCTGGAA-. . .GCCCTTT	176
Pf-5/1-941	121	TTTCGACAGCCTG	TTCGCGGACGATCGCCC-GGGGGCGGTCAGCGATGACGCGGCCCGT	180
15G2/1-941	177	GTGCGGTGT	GCTGTGCTGCTGTCAGCGCGGGCCAGGCGGGCCAATCAGCCC-TGT	236
Pf-5/1-941	181	GGACGGTAT	TCGGGATCTTCGTGCGCACCCCTGA-GCAACGACGGCCAAGTTGGCCCTGA	239
15G2/1-941	237	TCCGGAAGAAAGG	CGGCATCGCCCGGTGTGAAGGGAGCTGTTCTGTGCAATGACGGCT	297
Pf-5/1-941	240	GGACCGCAGCTA	CCTGGCG-DAATTGGTGGCCAGCGTADCAA-CCTCAGCCAGGCCGATG	298
15G2/1-941	298	CCATCAGCGCT	TCGAAGAAGAACTGCGCGTGAATGTTGGCCAAAGCTCCTCCACC	358
Pf-5/1-941	299	CCG--AGCGT	CGGGTCGATGAAGTCTTCGCCAAGACCAGCCAGGCGCTGGAAGAAGCCAA	357
15G2/1-941	359	C-. . . .CGCGAGCC	-GCTGCTG-DGTGATACDCAAGGGTGGC-ACCTG-CGGCAGCGCAGT	410
Pf-5/1-941	358	CAGGCCGCCAAG	GAAAGCTGCCGACACCAGCCAGGAGGTGGCTGCTGCTGCTGCTGGA	418
15G2/1-941	411	TTCTGCACCGGGC	CAAGGGCGGGGCTATTCGCTGACGCCCAGTGGCAACAAGAGCTGCA	471
Pf-5/1-941	419	TGTT-TATCGCGT	CTGTGCGGGGCTTCTTCGCCA-GCCTCGCGGCGACCTTTG-. . .GCG	475
15G2/1-941	472	AGCGTCGTTAGG	-CTCTGTACCAAGCGTTTTGAGACGAAGCTCAGGCAAGGGCGAAAACGGT	531
Pf-5/1-941	476	GTGTCGTCGGG	ACGCGTGGTCTG-GCTTGAAGCGATGTCAGCGCGCCACCGTAGCC	535
15G2/1-941	532	DGAGGAACGGCC	GGGGTCGCGTTCGACTTTAGGGTTGTAAACAGTGCCAATGTTATAAAG	592
Pf-5/1-941	536	DCAACCCCGTT	CACCTGATCAGGAGATTTCCATGCGCTCAATTACTCTTGTTCCCTCG	596
15G2/1-941	593	TTCCGGCGGCTGT	CTCGATGCTDTGAACCACTAATTGGGACAGGGCCCTGACCTTTGGC	653
Pf-5/1-941	597	TGTGC-CGAT	CCCCGGTGAATTATCTGATCGCCCTGTTTCATG-CACTGATTCAGCTATAAA	655
15G2/1-941	654	GGGGTTCGGC	AGGGCCGTTGGTATGCTTAAAGGCGCGCTGCCGGGCTTTCGCCGGCCG	714
Pf-5/1-941	656	CCCGGCCCGAAC	GGGCCCGGGCCTTCCCGAA-. . .CGCCGCTGCCGG-CCATGCCCAG	712
15G2/1-941	715	CGGCTTTTTGT	GCGTG-TATCGCAAGTTT-CAGAGADAAAGCACTGCTTTGAGGACACC	773
Pf-5/1-941	713	CGGCTTTTTGT	GCGCGCTATCGAGTTTGCAGGGCTGGCCACCCTCGGTCGAGGCA	773
15G2/1-941	774	GTCTTTTGTG	CTTGGCCAGATCAATATTTCTGTGACACAAAACACTAGATTTGCCCGCG	834
Pf-5/1-941	774	G-CCGTTT	GCTTGGCCAGATCAATATCCGTGTGACACAAAACACTAGATTTGCCCGCG	833
15G2/1-941	835	TCGGTTCGACAGC	CACTTCGGAACCGCGGTGTGACCGCGTCCCTTGGCCAGGACTCCAGG	895
Pf-5/1-941	834	TCGGTTCGACAAA	CAGCTTGGGACCGGACCGTTGCGCGTCCCGACCGDAGCAGCATGGA	894
15G2/1-941	896	DGGCAGGAT	GCCGGTAAACCCACATCACTTCTCACGGATGAACC-GCA	941
Pf-5/1-941	895	DGGCGGAGC	GCCGGCAACCCCATTCATTTTCACGGATGAACCACA	941

**Figure 3.12** Nucleotide sequence alignment of 5' flanking sequences of the *hcnABC* gene cluster of *Pseudomonas sp.* strain 15G2 and *P. fluorescens* strain Pf-5. The identity in nucleotide sequences between bacterial strains is approximately 53%. The alignment was made by the ClustalW2 program (<http://www.ebi.ac.uk/Tools/clustalw2/index.html>). Identical residues are shaded blue.

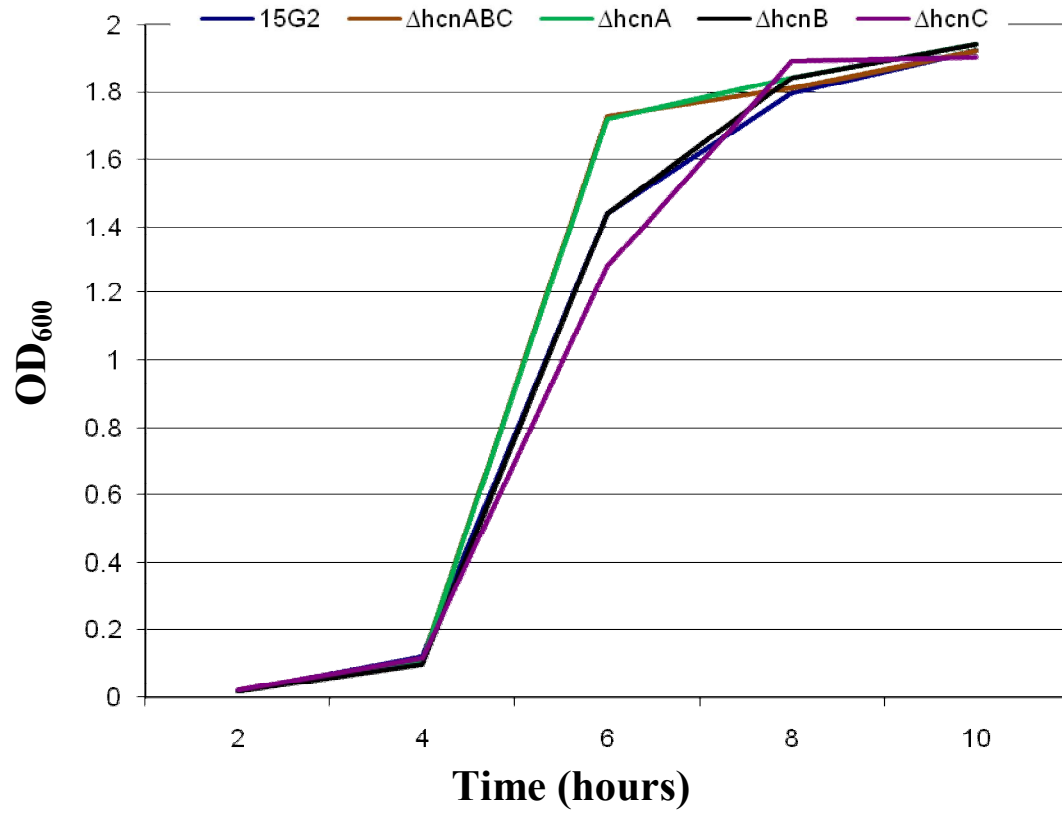


**Figure 3.13** Nucleotide sequence alignment of 3' flanking sequences of the *hcnABC* gene cluster of *Pseudomonas sp.* strain 15G2 and *P. fluorescens* strain Pf-5. The identity in amino acid sequence between bacterial strains is approximately 63%. The alignment was made by the ClustalW2 program (<http://www.ebi.ac.uk/Tools/clustalw2/index.html>). Identical residues are shaded blue.

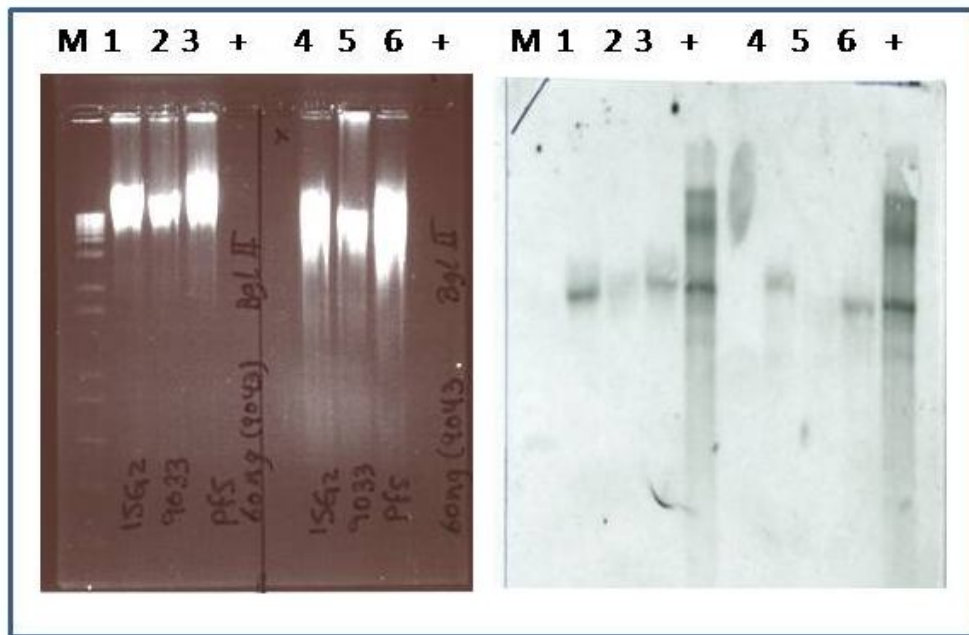




**Figure 3.14** Cyanide production by wild type *Pseudomonas sp.* strain 15G2, 15G2-*hcn* mutants, and 15G2-Tn5 mutants. Cyanide concentrations (µM/mL) produced by LB grown cultures and trapped in NaOH was measured after 3 days at 30 °C.



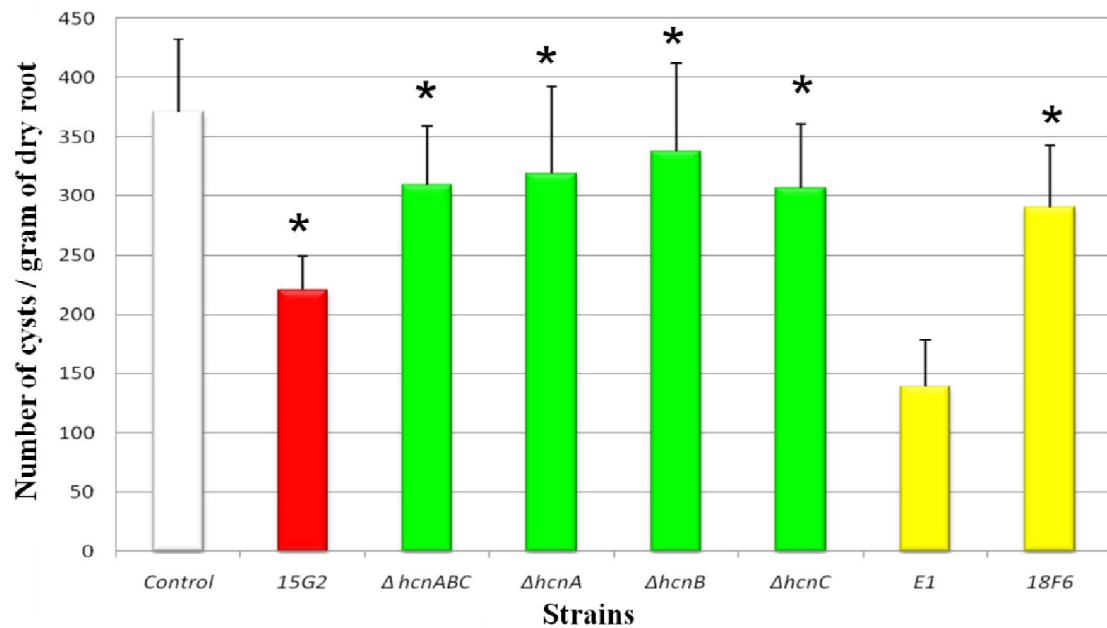
**Figure 3.15** Growth rates of *Pseudomonas* sp. strain 15G2,  $\Delta hcnABC$ ,  $\Delta hcnA$ ,  $\Delta hcnB$ , and  $\Delta hcnC$ .



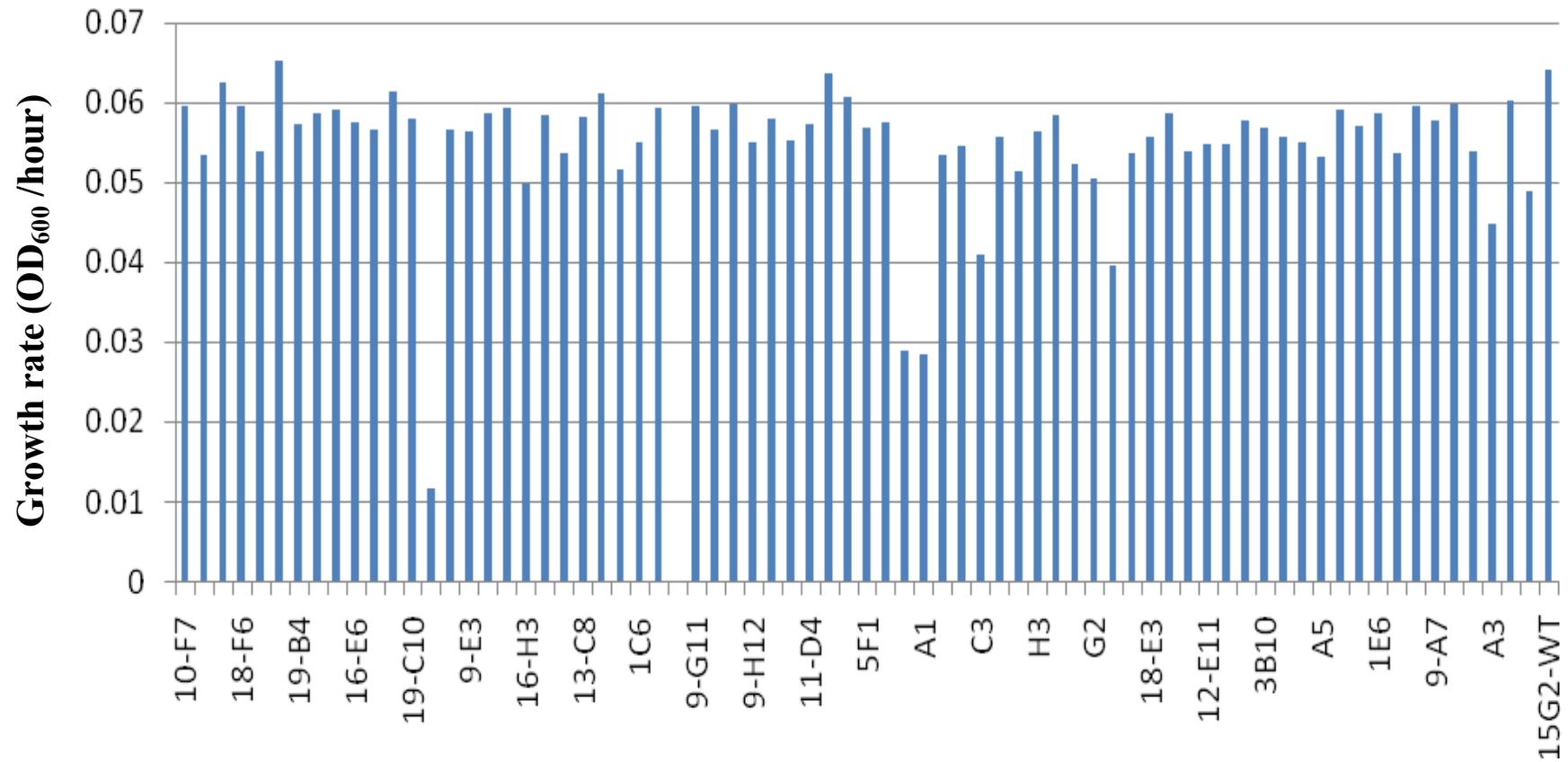
**Figure 3.16** Restriction digestion (A) and Southern blot hybridization (B) analysis of total DNA from *Pseudomonas* sp. strain 15G2, 15G2- $\Delta$ *hcnABC*, and *P. fluorescens* strain Pf-5. Three micrograms of total DNA from each strain were digested with *Eco*RI (Lane 1, 2, and 3), or *Nco*I (Lane 4, 5, and 6). Sixty ng of plasmid harboring *hcnB* gene were digested and used as a positive control (Lane +). Lane 1 and 4 represent strain 15G2 digested DNA; lane 2, and 5 represent 15G2- $\Delta$ *hcnABC* digested DNA; and lane 3 and 6 represent Pf-5 digested DNA. The digested DNAs were analyzed on 1% agarose gel, transferred to nylon membrane, and hybridized with the *hcnB* probe (650bp).

### 3.3.8 Hydrogen cyanide produced by strain 15G2 contribute to bacterial lethality toward plant-parasitic nematodes

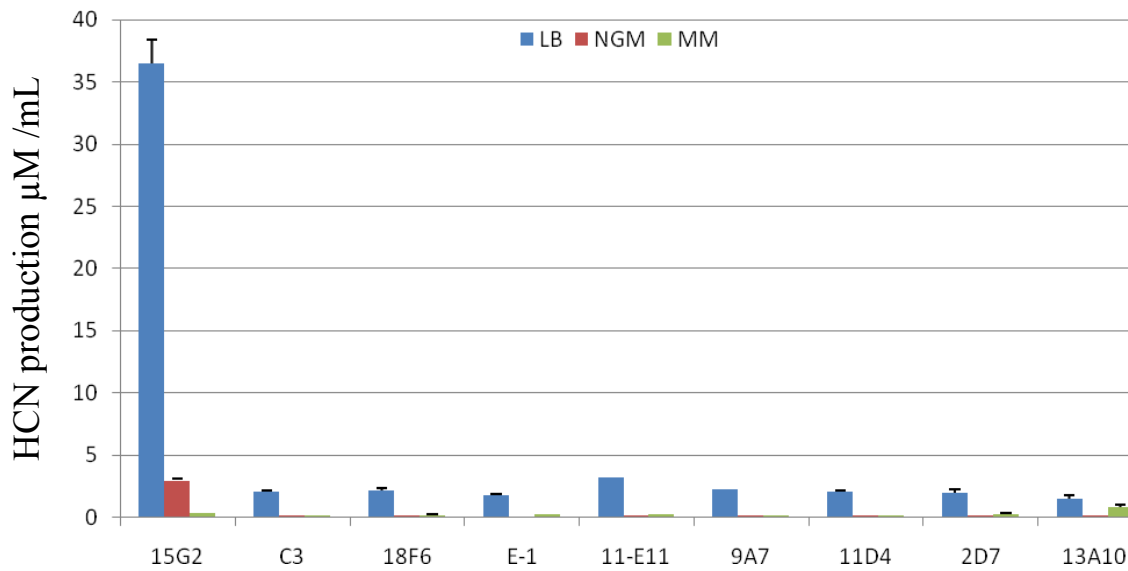
Our previous data (see *Chapter II*) demonstrated the ability of *Pseudomonas* sp. strain 15G2 to antagonize and reduce the plant-parasitic nematodes densities *in vitro* and *in vivo*. The influence of the hydrogen cyanide produced by strain 15G2 on its biocontrol abilities toward plant-parasitic nematodes was assessed in unsterilized sandy soil. In these experiments, we investigated the effect of the HCN-producing strain 15G2 and HCN-negative strains,  $\Delta hcnABC$ ,  $\Delta hcnA$ ,  $\Delta hcnB$ ,  $\Delta hcnC$ , and two of the 15G2-Tn5 mutants (*E-1* and *18-F6*) on the soybean cyst nematode (SCN) populations. Ten-day-old soybean seedlings were inoculated each with  $10^4$  bacterial cells per gram of soil. Three days later, 4000 soybean cyst nematode eggs were inoculated into the soil. After 30 days, the cysts were collected and root systems were blotted dry. The results showed that the *Pseudomonas* sp. strain 15G2 significantly ( $P < 0.05$ ) reduced the number of cysts (Figure 3.17). Mutants  $\Delta hcnABC$ ,  $\Delta hcnA$ ,  $\Delta hcnB$ ,  $\Delta hcnC$ , and *18-F6* failed to significantly reduce the number of cyst nematodes (Figure 3.17). These results clearly demonstrated the role of the HCN as an important antagonistic factor in nematode development.



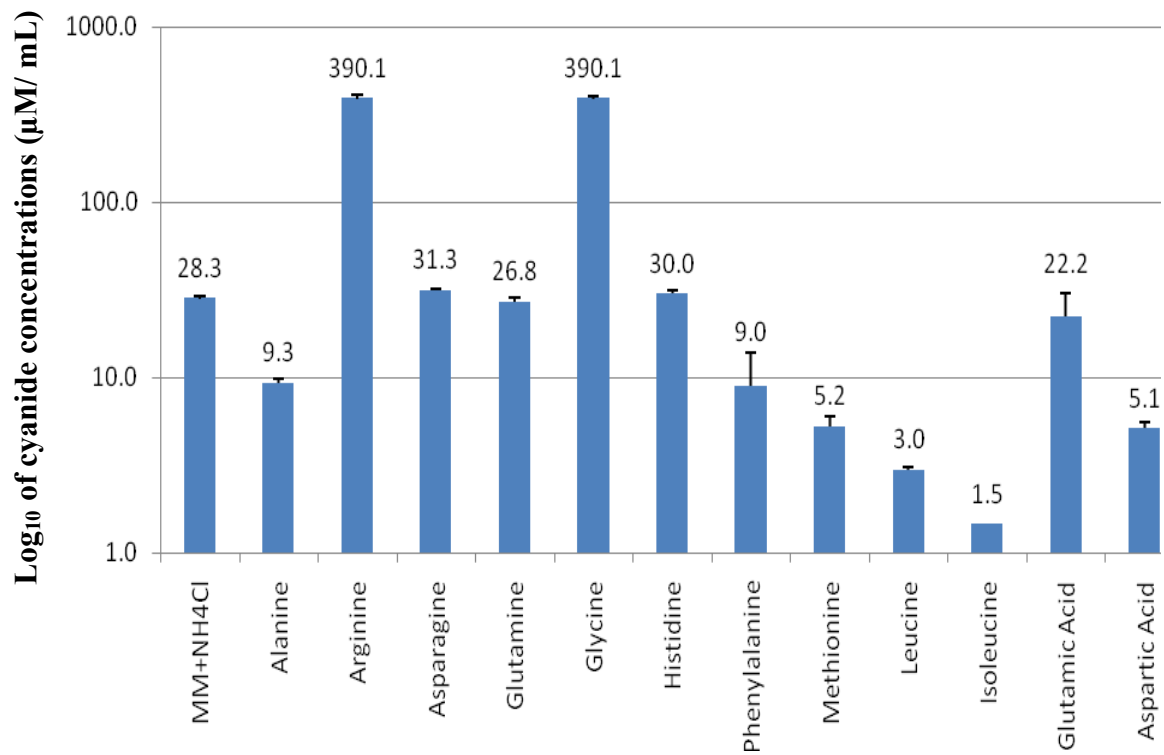
**Figure 3.17** The effects of the wild type *Pseudomonas sp.* strain 15G2 and its *hcn* mutants on the reproduction of the soybean cyst nematode (*Heterodera glycines*). The average of three independent trays (15 plants each), inoculated with each strain, is shown. Bar with each column represents the standard error. E1 and 18F6 are Tn5 mutants. \* indicates the significance of the results ( $P < 0.005$ ).



**Figure 3.18** Growth rates of wild type strain 15G2 and transposon mutants on LB media.



**Figure 3.19** HCN production by WT strain 15G2 and 15G2-transposon mutants growing on LB, NGM, and MM media. Cyanide concentrations ( $\mu\text{M}/\text{mL}$ ) produced by grown cultures and trapped in NaOH were measured after 3 days at 30 °C.



**Figure 3.20** HCN production by WT strain 15G2 growing on MM media supplemented with 1 µM of different amino acids.



### 3.4 Discussion

In this study, we provide further insights into the role of HCN plays as a control mechanism by *Pseudomonas* sp strain 15G2 to antagonize plant-parasitic nematodes and how the potential application of this strain broadens the scope of options for nematode management. Our results demonstrated a crucial role of HCN in killing nematodes *in vitro* and *in vivo*. Indirect exposure of both *C. elegans* and root-knot nematode juveniles to HCN produced by *Pseudomonas* sp. strain 15G2 rapidly paralyzed and killed the nematodes *in vitro* (Figure 3.5). A number of generated transposon insertion mutants as well as mutations in the *hcnABC* structural genes produced little to no detectable amount of HCN (Figure 3.14). These mutants failed to paralyze and kill the nematode *in vitro*. Similarly, exposure of *C. elegans* to exogenous cyanide at levels equivalent to those produced by *Pseudomonas aeruginosa* PAO1 rapidly killed the nematode (Gallagher and Manoil, 2001). In addition, the exogenous exposure to KCN significantly inhibited egg hatching and induced mortality of *M. hapla* eggs *in vitro* (Siddiqui et al., 2006). In our experiments, we found that direct exposure of both *C. elegans* and root-knot nematode juveniles to 15G2 mutants (transposon and *hcnABC* mutants) resulted in killing the nematode but over a longer time period (10 days). We concluded that if HCN is present, the effect on killing the nematode occurs in a matter of hours. However, if HCN is absent, other substances are built up in the medium and lead to the nematode dying over a longer time period.

The role of the HCN produced by strain 15G2 in the biocontrol of plant-parasitic nematodes was confirmed in soybean *in planta* bioassays. The WT strain 15G2, which produces a considerable amount of HCN, significantly ( $P < 0.05$ ) reduced the disease

development by soybean cyst nematode, whereas the HCN defective mutants lost most of their nematode suppression abilities (Figure 3.17). Together, the characteristics and abilities of strain 15G2 make this bacterium a successful candidate for the pathogen biocontrol.

A role for the HCN produced by *Pseudomonas fluorescens* strain CHA0 on the inhibition of root-knot nematode egg hatching has also been documented by others (Siddiqui et al., 2006). Another study has shown that incorporation of sudan grass leaves, which contain a considerable amount of cyanide, significantly affects the egg development and reduces the root-gall-severity of the root-knot nematode, *M. hapla* (Widmer and Abawi, 2002). Furthermore, the role of cyanide in suppressing other plant diseases such as plant fungal diseases caused by *Thielaviopsis basicola* (Voisard et al., 1989) and the plant termite, *Odontotermes obesus* (Devi et al., 2007), has been recognized.

Cyanide is a secondary metabolite produced by several types of bacteria such as that produced by protobacteria: *P. fluorescens*, *P. aeruginosa* (Askeland and Morrison, 1983), and *P. entomophila* (Ryall et al., 2009). In these strains, HCN and CO<sub>2</sub> are produced through the oxidation of glycine by hydrogen cyanide synthases (Blumer and Haas, 2000). In several *Pseudomonas* species, three structural genes, *hcnABC*, have been recognized to encode for hydrogen cyanide synthases, and these genes share sequence similarities with known amino acid dehydrogenases (Blumer and Haas, 2000). Our southern blot analysis revealed that strain 15G2 possesses one copy of the *hcnABC* gene cluster similar to that of strain Pf-5. Deduced amino acid sequences of strain 15G2 *hcnABC* were closely related (94%) to that of strain Pf-5 (Figure 3.11). However, the 5'

and 3' *hcnABC* flanking sequences shared 53 and 63% homology, respectively, with that of strain Pf-5 (Figure 3.12 & 3.13). These results may suggest differences in the *hcnABC* locus in both strains and could explain why we could not amplify the 15G2 sequences using primers designed at the *hcnABC* flanking region according to the Pf-5 strain sequence.

Early physiological experiments in *P. aeruginosa* have shown that HCN production depends on the cell densities and oxygen levels, and maximum production occurs during the transition between the exponential and stationary phases (Castric et al., 1979; Castric, 1983; Castric and Deal, 1994). A similar HCN production pattern was observed for our strain 15G2 (Figure 3.4). The cyanogenic capacity of strain 15G2 grown on different media was determined. The greatest HCN production was obtained when bacteria were grown on LB media (Figure 3.19), whereas the production was significantly reduced when grown on MM and NGM media. A possible explanation for the variation of HCN production on different culture media could be the differing factors used to enhance HCN production, such as amino acids and iron, in the media. However, individual addition of different amino acids such as glycine, arginine, asparagine, histidine, and glutamic acid to the minimal media enhanced the ability of the strain 15G2 to produce HCN (Figure 3.20). The addition of different amino acids such as glycine, methionine, leucine, tyrosine and histidine enhanced the HCN production by *P. fluorescens* (Castric, 1977; Askeland and Morrison, 1983).

Hydrogen cyanide is a potent toxin that binds to ferric iron containing enzymes such as cytochrome oxidases and other metalloenzymes, thus inhibiting mitochondrial respiration (Way, 1984; Blumer and Haas, 2000). In *P. aeruginosa* and *P. fluorescens*,

HCN production is controlled by the GacA activator and regulated by the quorum sensor regulators LasR and RhlR, and anaerobic regulator ANR (Blumer and Haas, 2000). The LysR family (transcriptional regulators) plays an important role in *P. aeruginosa* pathogenicity via the transcriptional regulation of several quorum-sensing- regulated virulence factors such as hydrogen cyanide (Xiao et al., 2006). In our study, mutations in 15G2-LysR transcription regulators (strains 12D12, 9E3, and 1C6) significantly reduced the HCN production (Table 3.3).

The use of transposon mutagenesis has been a powerful tool in functional analysis of bacteria. In this study, nearly 2,700 transposon mutants for strain 15G2 were generated. Out of these transposon mutants, 74 showed impaired activities against *C. elegans*. The transposon insertions affected several genes that encode for different cellular metabolic enzymes, transporters, proteases, regulators, and other genes with no assigned function (Table 3.3). Fourteen mutants with growth rates comparable to those of the WT showed reduction in their HCN production (Figure 3.14). Interestingly, none of these transposon insertions were in the *hcnABC* locus or any other genes that encode for HCN regulators, which may be due to the multiple hits of several genes we obtained and/or the effects of polarity and insertional randomness of the transposon. Among these transposon mutants defective in killing *C. elegans*, three mutants (*16-C8*, *9-H12*, and *18-A1*) affected genes encoding for periplasmic subunits of amino acid transporters. Our results showed that the complementation of these mutants with the corresponding WT genes restored their ability to produce HCN at levels close to that of strain 15G2 (Figure 3.8). We do not know at this time how these genes affect HCN production. However, HCN production has been suggested to take place in the periplasmic region (Blumer and

Haas, 2000). In addition, a recent study by Cipollone et al. (2006) showed that the cyanide detoxification mechanism by the *P. aeruginosa* enzyme, rhodanase, occurs in the periplasm. Our results clearly imply that these amino acid transporter proteins may play a role in hydrogen cyanide production through delivering amino acids to the periplasm to be used as a precursor for HCN production. Considering this evidence, we suggest that these periplasmic subunits are located in close proximity to HCN synthases and transport the amino acids required for HCN synthesis. Four other mutants, *18-F6*, *G3*, and *A1*, encode for a dehydrogenase enzyme. They show reduction in their ability to produce HCN and kill the nematode *in vitro* and *in vivo* (Figure 3.5 & 3.17). A transposon mutation in the *zwf* gene, which encodes for glucose-6-phosphate dehydrogenase of the *P. aeruginosa* PAO1, reduced its ability to kill *C. elegans* by 60% (Gallagher and Manoil, 2001).

The amino acid sequencing analysis of HCN synthases showed similarities with known dehydrogenases; HcnA with formate dehydrogenase, and HcnB and HcnC subunits with amino acid dehydrogenase oxidases (Laville et al., 1998; Blumer and Haas, 2000). These analyses suggested that HCN synthases function as amino acid oxidases. Our results showed that strain 15G2 has the ability to produce HCN from nine different amino acids: alanine, arginine, asparagine, aspartate, glutamate, glutamine, histidine, proline, and serine. Further research is needed to determine if HCN is being produced directly or indirectly from these amino acids. It is not unlikely to expect that bacteria could use a different pathway to produce hydrogen cyanide. All HCN mutants generated in this study and by other laboratories did not result in 100% loss of HCN production. This may account for the low level (< 5  $\mu$ M) produced by  $\Delta hcnABC$  mutants. Our results

showed that strain 15G2 can use different amino acids for growth and, thus, HCN production (Figure 3.7). In some cyanobacteria, hydrogen cyanide can be produced by histidine oxidation pathway (Pistorius et al., 1979; Gewitz et al., 1980).

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## **CHAPTER IV**

**Transcriptional profiling of plant responses  
to cyanide: relevant biomarkers for bacterial  
cyanide detection in soil**

## Contribution of Authorship

All experiments, figures, figure legends, and Materials and Methods selection were conducted or created by Haytham Aly. The microarray analysis was done at MOgene Company (St. Louis, Missouri) and the statistical analysis method was written and performed by Drs. Lauren McIntyre and Alison Morse (University of Florida).

### 4.1 Introduction

The phenomenon of cyanogenesis has been reported to occur in several species of bacteria, fungi, algae, insects, and plants (Uecker and Blumel, 1968; Knowles, 1976; Vennesland et al., 1981; Knowles, 1988). Bacterial cyanogenesis has been recognized in different protobacteria, such as *Pseudomonas aeruginosa*, *P. fluorescens*, *P. entomophila*, *P. aureofaciens*, *Burkholderia cepacia*, and *Chromobacterium violaceum* (Rodgers and Knowles, 1978; Askeland and Morrison, 1983; Gallagher and Manoil, 2001; Ryall et al., 2008; Ryall et al., 2009). Also, cyanogenesis has been reported in some cyanogenic bacterial families such as *Anacystis*, *Nostoc*, and *Plectonema* (Knowles, 1988). *Pseudomonas* spp. are abundant in the agricultural soils, known for their excellent colonization of the rhizosphere, have the ability to promote plant growth and have the potential to produce secondary metabolites (such as DAPG, siderophores, phenazines, and/or HCN), which accounts for their biological control abilities (Haas et al., 2000). A role for HCN produced by the *Pseudomonas* spp. in the biocontrol of different plant-pathogens such as fungi, pathogenic bacteria and nematodes has been suggested by others (Gallagher and Manoil, 2001; Siddiqui et al., 2006; Voisard et al., 1989). Environmental conditions such as availability of iron, phosphate, amino acids and pH affect cyanide

production by these bacteria. For example, optimum cyanide production occurred in the presence of glycine, when the pH is between 6.6 and 8.9 and available phosphate concentration was  $>300$  mM (Askeland and Morrison, 1983). Cyanogenesis by *Pseudomonas* is inhibited under iron-depletion conditions (Blumer and Haas, 2000).

In higher plants, cyanide is produced either as hydrogen cyanide during ethylene biosynthesis or by the hydrolysis of cyanogenic glycosides (Peiser et al., 1984; Piotrowski and Volmer, 2006). There are over 2000 plant species that naturally produce cyanogenic glycosides as products of secondary metabolisms and store them in their vacuoles. Upon ingestion by insects or small herbivores, the plant releases the cyanogenic glycosides, which then get hydrolyzed by  $\beta$ -glycosidase to release HCN (Tattersall et al., 2001). The oxidation of the ethylene intermediate precursor, 1-aminocyclo-propane-1-carboxylic acid (ACC), by ACC oxidase forms one molecule ethylene and one molecule cyanofomric acid. The latter is an unstable molecule that is spontaneously degraded to form hydrogen cyanide and carbon dioxide (Peiser et al., 1984). The accumulation of cyanide in plant cells leads to toxicity and cell death. However, plants naturally possess the ability to cope with this endogenous cyanide. Detoxification of cyanide occurs via the  $\beta$ -cyanoalanine pathway in which the cyanoalanine synthase catalyzes the interaction between HCN and cysteine to form  $\beta$ -cyanoalanine, which is then converted by cyanoalanine hydratase to ammonia, asparagine, and asparatic acid (Figure 4.1) (Jenrich et al., 2007).

Several studies have investigated the biological role of cyanide in higher plants and suggested that it may play a beneficial role in plant growth and development. For example, cyanide directly breaks seed dormancy and induces germination (Hasegawa et



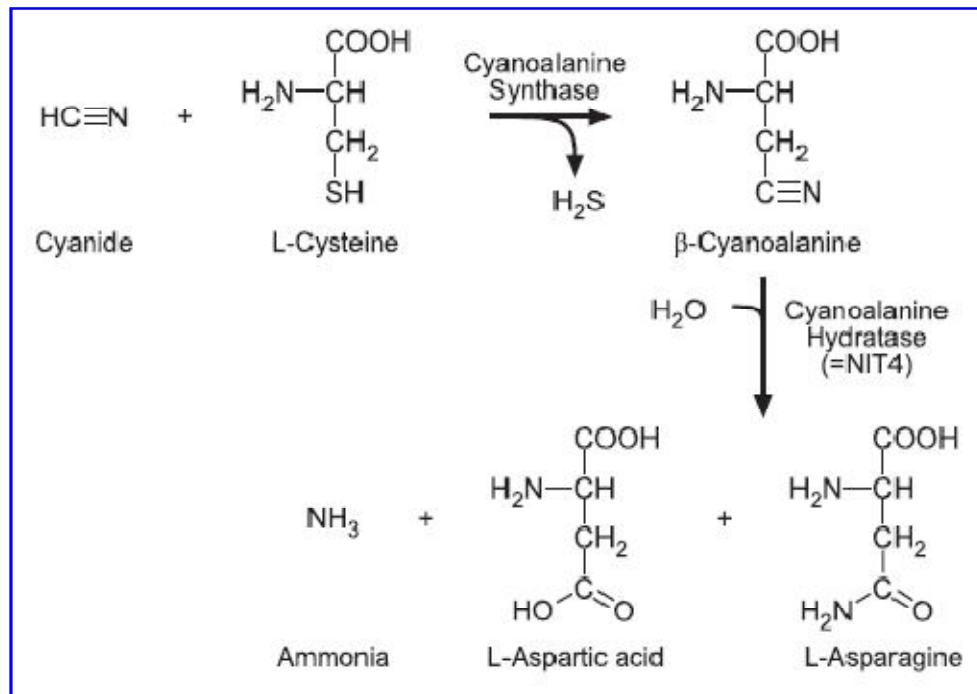
al., 1995). Similarly, stimulation of cocklebur seed germination by cyanide treatment leads to accumulation of total amino acid in seeds, possibly through the  $\beta$ -cyanoalanine pathway (Maruyama et al., 1996). Also, cyanide accumulation may increase plant resistance. For instance, upon infection with tobacco mosaic virus, cyanide accumulates in the infected cells leading to toxicity and cell death, thus protecting the plant from disease development (Siefert et al., 1995).

On the other hand, cyanide may negatively affect the growth of some plant species. The cyanide treatment of *Arabidopsis* plants caused reduction in the chlorophyll content and resulted in stunted growth (Smith and Arteca, 2000). In addition, this treatment increased the expression of *ACS6* (1- aminocyclopropane-1-carboxylic acid synthase) gene that produces 1- aminocyclopropane-1-carboxylic acid (ACC) from adenosylmethionine, suggesting a possible role of cyanide in controlling the ethylene pathway. Another study has shown that direct exposure of transgenic *Arabidopsis* plants (expressing a GUS reporter gene driven by auxin responsive promoter DR5 (DR5::GUS) to KCN or indirectly to cyanide produced by *P. aeruginosa* caused growth inhibition through the suppression of auxin production and/or transport (Rudrappa et al., 2008). HCN-defective bacterial strains do not affect plant growth suggesting that cyanide may interfere with the auxin signaling pathway leading to growth inhibition.

To date, no study has looked at the full gene expression profile of plants associated with cyanide treatment and how cyanide affects different biological processes. Very few studies have attempted to elucidate the effects of bacterial cyanide in the rhizosphere on plant growth and development. Further studies are needed to provide more insights about the natural interactions that occur between plant and cyanide

producing bacteria and determine the factors that lead to negative or positive effects on plant growth.

The aims of the present study are to (i) conduct a microarray analysis of *Arabidopsis* gene expression during cyanide treatment to highlight the importance of critical biological processes and identify major gene-expression patterns in response to cyanide exposure; (ii) confirm the microarray results using quantitative PCR analysis and available knockout mutants; (iii) identify a set of genes that can be used as biomarkers for the cyanide response; (iv) Evaluate the biomarkers by using the HCN- producing *Pseudomonas* sp. strain 15G2 and the HCN-nonproducing strain 15G2- $\Delta hcnABC$  in *in vitro* and *in vivo* experiments.



**Figure 4.1** Cyanide detoxification pathway (Jenrich et al., 2007).

## 4.2 Materials and Methods

### 4.2.1 Plant materials and growth conditions

*Arabidopsis thaliana* ecotype Columbia (Col-0) seeds, transgenic *Arabidopsis* lines expressing ACS6-GUS/GFP and *Arabidopsis* SALK lines (020571C, 068280C, 142404C, 054530C, 017254C, 014515C, 091130C, 039038C, 039436C, 015916C, 107806C, 092291C, 052858C, 007343C, 031983C, and 124967C) were obtained from the *Arabidopsis* stock center at the Ohio State University. Transgenic *Arabidopsis* line stably expressing DR5::GUS was obtained from Dr. Thomas Guilfoyle, University of Missouri Columbia, Missouri. CASase mutant, Bsas3;1, was obtained from Dr. Mutsumi Watanabe, Chiba University, Japan. Seeds were surfaced sterilized by 70% ethanol and 2.5% sodium hypochloride for 10 min followed by 3 washes in sterilized water. The seeds were sown on Gamborg's B5 basal medium (Gamborg et al., 1968), placed at 4°C for 2 days and then transferred to a growth chamber at 22°C and 60% relative humidity under white fluorescent light. For the greenhouse experiments, seeds were grown in Fafard superfine germination media (B&T Grower supply, Inc) in 3-inch pots and put in a growth chamber at 22°C under 12-h light/dark cycle. The light level was maintained at  $200 \mu\text{mol m}^{-2} \text{s}^{-1}$ .

### 4.2.2 Cyanide treatments and hydrogen cyanide quantifications

All cyanide degassing treatments were occurred in closed 10 L dessicator cabinets without using any vacuum. To determine the cyanide degassing rate and the atmospheric HCN inside the dessicator, four different concentrations of KCN (100, 200, 400, and 600 mM) were dissolved in 0.09M NaOH and tested. Each NaOH-buffered KCN solution

was then poured in a deep petri dish with a magnetic stirrer and placed in the bottom of the dessicator. On the shelf inside the dessicator, three opened petri dishes each containing 4 mL of 5M NaOH, were sited and the dessicator was then placed on a stirrer plate. To degas the KCN, 0.018 M HCL was added rapidly to the KCN solution and the dessicator was closed. After 2 hours, the cyanide contents in the 4 mL NaOH, which correlate to the atmospheric HCN captured, was quantified as described in *Chapter II* using cyanide-ion-selective electrode.

### 4.2.3 Microarray

**Experimental set up.** Three-week old *Arabidopsis* plants (Col-0) grown axenically in petri plates (five plants/plate) were used for the cyanide treatment and microarray experiment. Experiments were performed similar to that of the KCN degassing experiment described above, except that petri plates containing the *Arabidopsis* seedlings were placed open on the shelf inside the dessicator instead of the 4 mL NaOH. The plants were challenged with 18  $\mu$ M of HCN (sublethal level of cyanide) for two hours. A mock-challenge (HCL added to NaOH) was also generated for use as a control. Four replicates were generated for both challenge and control. Five plants (one plate) were combined for RNA extraction to make a single treatment.

**RNA extraction and analysis.** Following the challenge, total RNA was extracted from excised whole root tissues using the RNeasy Plant mini kit (Qiagen) and treated with RQ1 RNase-free DNase (Promega) following the manufacturer's instructions. Total RNA samples were sent to the microarray facility at MOgene, LC Biotechnology Company for cDNA and cRNA synthesis, fragmentation, hybridization to

the Affymetrix Arabidopsis Genome Array chip (Agilent, Arabidopsis Array 4), staining and scanning. Four arrays were analyzed using two of the replicates in a dye swap.

#### 4.2.4 Statistical analysis of microarray data

Images were analyzed using the Agilent Feature Extractor at MOgen. The mean intensity for each quantified spot and the corresponding mean background signal was exported into a '.txt' file. Individual files were collated for analysis. Transcript abundance was estimated as the natural log of the spot mean minus the mean of the local background. There were 1417 Agilent control, on the array leaving 43,803 features for analysis. A feature was said to be detected if the intensity at the probe position was in the top 20% for at least one of the hybridizations. A total of 36,839 probes were detected in at least one treatment. While this method may eliminate from consideration some features with low levels of expression, it will not eliminate any features for which change in relative expression is different between the HCN treatment and the control samples. A large dye bias was observed and dye variances also appeared different. The experiment performed was balanced over dyes such that two HCN and two control samples were hybridized for each dye. In order to further reduce the impact of the dye, samples were rank transformed.

Each feature was identified by a corresponding gene in the manufacturer's annotation. There were 7975 genes that had a single feature for analysis and 25166 genes that had more than one feature. For each gene the following model was fitted:

$$Y_{ijkn} = \mu + d_i + t_j + a_k + \varepsilon_{ijkn} .$$

$Y_{ijkn}$  is the transcript abundance for the  $n^{\text{th}}$  replicate,  $\mu$  is the overall mean,  $d$  is the effect of dye ( $i = 1, 2$ ),  $t$  is the effect of treatment ( $j = 1, 2$ ),  $a$  is the

random effect of array and  $\mu$  is the error. As type I and type II errors are inversely related, we decided to minimize the chance of making a type II error in this stage of analysis at the increased risk of having probes included that were in fact type I errors. Accordingly, all probes significant at a False Discovery Rate (FDR) of 0.2 for treatment were retained for further analysis (Benjamini and Hochberg, 1995; Koen et al., 2005).

The features on the array were BLASTed against the Arabidopsis genome in TAIR v8. Where possible, features were identified with a locus by TAIR. There are 3736 features on the chip with no match or no known locus, likely due to the inclusion of features on the array from EST projects not yet fully annotated by TAIR. When a gene, as identified by Agilent, has more than one locus this is noted. TAIR also provides a Gene ontology annotation for each locus. For some loci more than one set of GO terms are associated with the locus. These are also noted. Loci from TAIR and their corresponding GO annotations were linked to genes using the feature identifiers provided by both Agilent and TAIR. For all genes analysed, the set of genes significant based on the above criteria were tested for enrichment using a Fisher's exact test. GO categories overrepresented (using a nominal  $p = 0.05$ ) were further examined.

#### **4.2.5 Real-time quantitative PCR**

*Confirming the microarray data.* In order to confirm the microarray data, we chose 23 up-regulated genes for further qPCR analysis. The list of the genes and the primer sequences used for qPCR are provided in Table 4.1. For the microarray validation we used three biological replicates (control and HCN challenged) of *Arabidopsis* plants that were grown independently from those used for the microarray. The experiments

were similar to that used for microarray analysis. The total RNA isolation from root tissues and RNase-free DNase treatment were performed as described above. The purified RNA was quantified using the Nanodrop ND-1000 spectrophotometer (Agilent). Three hundred nanograms of total RNA were used for cDNA synthesis using the SuperScript III First-Strand Synthesis kit (Invitrogen) according to the manufacture's protocol. For quantification, transcript levels were assayed using Mx3005P- qPCR System (Stratagene). qPCRs were conducted in triplicates using gene specific primers (Table 4.1) in a 25  $\mu$ L reaction using the Brilliant™ SYBR® Green master mix (Agilent). PCR conditions used were 95°C for 5 min, followed by 40 cycles of 95°C for 15 sec, 55°C for 1 min and 72°C for 30 sec. The Arabidopsis housekeeping gene, Actin-2 (At3g18780) was used as an internal control for all the experiments conducted. We determined that expression of this internal control gene is not altered by cyanide or bacterial treatments.

*Validation of microarray results using gene knockout Arabidopsis lines.* To further confirm the gene response to cyanide treatment, we obtained 16 different SALK lines that were available for the highest up-regulated genes (see plant material section) and challenged them with 18  $\mu$ M cyanide for two hours in similar experiments to that described above. After cyanide treatment, plants were placed back in the growth chamber and their phenotype was monitored visually over 10 days.

*Cyanide-time point experiments.* In order to identify genes with early response to cyanide treatment, similar experiments were conducted and the cyanide treatment was performed at different time points. Three biological replicates were used and root samples were collected at 15 min, 30 min, 1 h and 2 h after cyanide exposure. In



addition, two 2 h treatments were performed followed by putting plants back in the growth chamber for 2 h (referred to as 4 h treatment) and for 22 h (referred to as 24 h). RNA extractions, qPCR analysis were identical to that described above.

**qPCR data analysis.** The qPCR data were analyzed using the relative quantification method  $2^{-\Delta\Delta C_t}$  according to (Livak and Schmittgen, 2001). We used the untreated control as the calibrator and calculated the fold change in the gene expression normalized to the *Actin-2* gene and relative to untreated control. The fold change in gene expression for untreated control is equal to one.

#### 4.2.6 Bacterial strain and culture conditions

The *Pseudomonas* sp. strain 15G2 was grown on Luria broth (LB) agar supplemented with 100 µg/ mL rifampicin. A hydrogen cyanide defective strain of the same bacteria, 15G2-*AhcnABC* was also grown on LB supplemented with 100 µg/ mL rifampicin and 50 µg/ mL kanamycin. To determine the presence and persistence of these strains in soil under our experimental conditions, colony forming units (CFU) per gram of soil were determined as described in *Chapter III*.

#### 4.2.7 Bacterial inoculations and plant bioassays

To study the effect of bacterial cyanide on *Arabidopsis* gene expression *in vitro*, two compartment petri dish plates were used as described by (Rudrappa et al., 2008) with some modifications. Briefly, one side of the two compartment plate was filled with Gamborg's B5 medium, and the other side was filled with LB agar medium. The indirect effect of bacterial cyanide was assayed first by growing five *Arabidopsis* seeds on the Gamborg's B5 medium and incubating them at 22°C for three weeks. Then, the LB side

was inoculated with 20  $\mu\text{L}$  of logarithmically growing bacteria ( $0.3 \text{ OD}_{600}$ ) and placed at  $22^\circ\text{C}$  for one day. The direct effect of the bacteria was assayed similarly, except that bacterial cells were collected by centrifugation and washed three times with distilled water, and then inoculated directly along the plant roots for two days.

To determine the effect of cyanide produced by strain 15G2 on *Arabidopsis* gene expression under soil conditions, logarithmically growing WT- 15G2 and 15G2-*ΔhcnABC* were collected and washed three times with distilled water and resuspended in water to a final concentration of  $10^4$  CFU/ mL. Three weeks old *Arabidopsis* plants were inoculated as a soil drench with these strains and placed in a growth chamber for one week. In all the assays described above, at the end of the experiment, roots of the *Arabidopsis* plants were collected and RNA extraction and qPCR analysis were performed as described earlier. For the qPCR data analysis, we used the 15G2-*ΔhcnABC* treated plants as the calibrator to eliminate the effects of the bacteria on gene expression and obtain the expression result from cyanide presence.

#### **4.2.8 Histochemical GUS assay**

Five-day old seedlings of the transgenic *Arabidopsis* lines DR5::GUS and ACS6-GUS/GFP were used to determine if they respond to cyanide produced by 15G2, and thus could be used as biomarkers for cyanide treatment. The experiments were similar to that described before in the two compartments plate assay except transgenic seeds were used instead of the WT *Arabidopsis*. The histochemical assay of  $\beta$ -glucouronidase (GUS) was performed as described (Jefferson et al., 1987). Treated seedlings were incubated overnight at  $37^\circ\text{C}$  in GUS staining solution (0.5 mM potassium ferrocyanide, 0.5 mM

potassium ferricyanide, 100 mM sodium phosphate (pH 7.5), 10 mM EDTA, and 1% TritonX-100 containing 1mM 5-bromo-4-chloro- $\beta$ -D-gluconide). After staining, tissue was cleared using two 70% ethanol washes. The GUS expression was visually observed.

**Table 4.1** Primers sequences designed for use in qPCR.

Gene ID	Primer name	Primer sequence (5' to 3')
At1g69930.1	DLS	F- TCAAGAATGCAGCAAAGGAA
	DLT	R- AATGTCGATGAACCCGATGT
At2g23170.1	DLU	F- CATCACAGAGTTCCTCACAAGC
	DLV	R- GTCGGTCCATGTCTTCATCA
At2g31860.1	DLW	F- GATTCAAGATCTGGTGTGACCT
	DLX	R- TGCTCATTCTTCCCCTGTT
At5g55150.1	DLY	F- TGGCAGTACACTAGGGTTTTTGT
	DLZ	R- AACCGCTGAACATCCTCAA
At1g07180.1	DQC	F- TTGATGCTGATAATCATGAGGTG
	DQD	R- CCAGTTTGTGATAAGCTATCTTGAAC
At1g29510.1	DQE	F- CATCAAAGTATCCAAAGACGA
	DQF	R- AGATTGACATCAACAGAGCCTTT
At1g29500.1	DQK	F- CTAATCAAGATGTTTCCAGGAAATGG
	DQL	R- CGGGTACTTGGTCTTTGGAA
At5g57520.1	DQM	F- TTGTTCTCGAGCCTTCTTCC
	DQN	R- AACATGATGATGAGTTCGTGGA
At1g14550.1	DQO	F- GACATCGACGCAGGATTTG
	DQP	R- CTGCCAGATTACCATCACCTC
At3g53770.1	DQQ	F- CGCTCGATCAACAACACAAT
	DQR	R- AAGTATCCGGTATCAGGGTCTG
At5g43450.1	DQU	F- CACTTGTTATATTGCTCCAGATCC
	DQV	R- TGTATTGATCACCCTGACTC
At1g75490.1	DQW	F- CTCATCAATCTCTCTGCTGGTTT
	DQX	R- TGTCTCGAGAGACCCTTCTTTT
At3g25760.1	DQY	F- AACTCCGGTACCACCGTCTA
	DQZ	R- GGCTTAAGCGCCTTAGCTTC
At5g40590.1	DRE	F- AGCTGCAATGTTAGAGGCTCA
	DRF	R- GCCAAATGCATCTCAATCTG
At2g36780.1	DRI	F- TCCAGGGGTATTATGGAAA
	DRJ	R- CTCCCACATGCGAGTAAGC
At5g07440.1	DRW	F- TCTTATATTTCCCAATATTTGTTTCCAGG
	DRX	R- GCCTCTACTATAAACTTTGCCTTCA
At1g37130.1	DRY	F- CTTTGGTAGACGCCGAATC
	DRZ	R- TTTAGCTCGTTGATTATGTAATCTGG
At5g22300.1	DTA	F- AGTACCATGCTTCTGCCATTG
	DTB	R- CCATTAACGCTAATCGTTCCA
At1g69850.1	DTC	F- GGCACAGCTTTTCTCCTAGC
	DTD	R- CCGAGCTTGAATTGTGAGTATG
At1g08090.1	DTE	F- GAAATCGAGCTACCTTGGAGAA
	DTF	R- TTGTAACGGCATAACCACAGAA
At1g12110.1	DTG	F- CCTTCTTGATGCTTGGGACT
	DTH	R- CTCCACGGCCTCAATACAA
At3g16150.1	DTI	F- TTCCGATCAGCATTACGC
	DTJ	R- CAATGTCCTTTCCCCTCAAC
At4g11280.1	DLM	F- TAGTCTTCTCCATAAATTTTCGCTACAG
	DLN	R- CTTTGTGGAGATTTGATGCGTAAATGGG
AtACT2	Actin-2F	F- GTTAGCAACTGGGATGATATGG
	Actin-2R	R- CAGCACCAATCGTGATGACTTGCCC

Symbols, F;forward and R; reverse

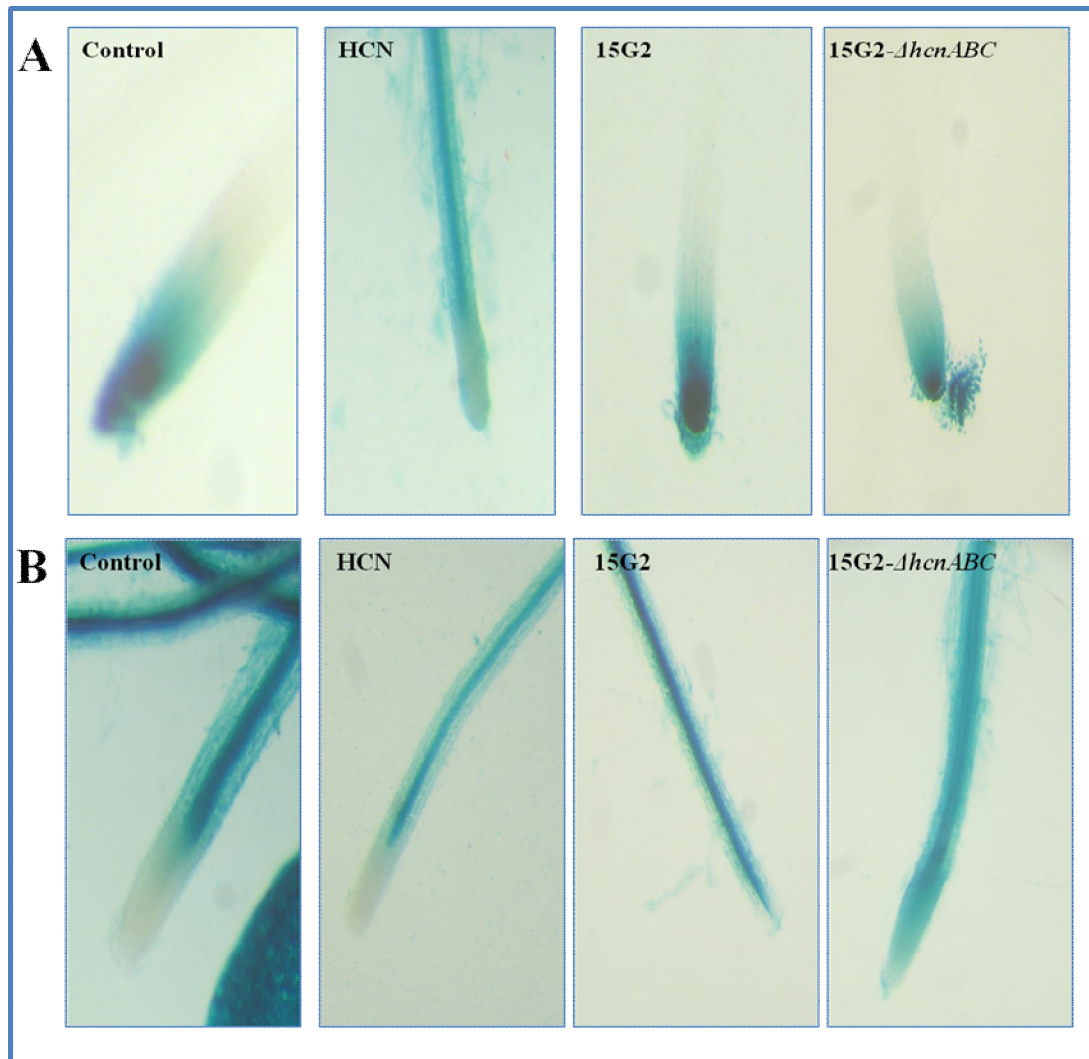
## 4.3 Results

### 4.3.1 *In vitro* analysis of the *DR5::GUS* and *ACS6* as biomarkers for cyanide treatment

The effects of cyanogenic *Pseudomonas* on plant growth were studied by Rudrappa et al., 2008. Their results showed that these bacteria inhibited plant growth and several HCN-producer strains of *Pseudomonas* caused significant reduction in primary root length. In addition, cyanide produced by *Pseudomonas* strains caused down-regulation of GUS expression when driven by an auxin responsive promoter *DR5*, suggesting a role of cyanide in inhibiting auxin biosynthesis. Based on these results, we decided to evaluate the direct and indirect effects of strain 15G2 on *DR5::GUS* expression and determine if we could use it as a biomarker for cyanide treatment. The results from our experiments showed that the treatment of *Arabidopsis* seedlings expressing *DR5::GUS* with KCN (>100  $\mu$ M) caused suppression in GUS expression at the root tips (Figure 4.2). However, when we used either strain 15G2 or 15G2-*AhcnABC* we obtained inconsistent results. Mostly, the expression of GUS was not suppressed at the root tips as shown by the other study (Figure 4.2A). Based on inconsistency of these results, we decided to not further test the *DR5::GUS* as a biomarker for cyanide treatment.

Another study has shown that treating *Arabidopsis* plants with either 50  $\mu$ M HCN for 20 min or 9  $\mu$ M for 40 min causes activation of the 1- aminocyclopropane-1-carboxylic acid synthase gene (*ACS6*) expression in leaves (Smith and Arteca, 2000). To evaluate *ACS6* as a possible biomarker for our study, we obtained transgenic *Arabidopsis* in which a *GUS/GFP* fusion is driven by *ACS6* promoter. Unfortunately,

the treatment of *ACS6::GUS/GFP* seedlings with KCN, strain 15G2 or 15G2- $\Delta hcnABC$  did not show any apparent differences between the treatments (Figure 4.2 B).



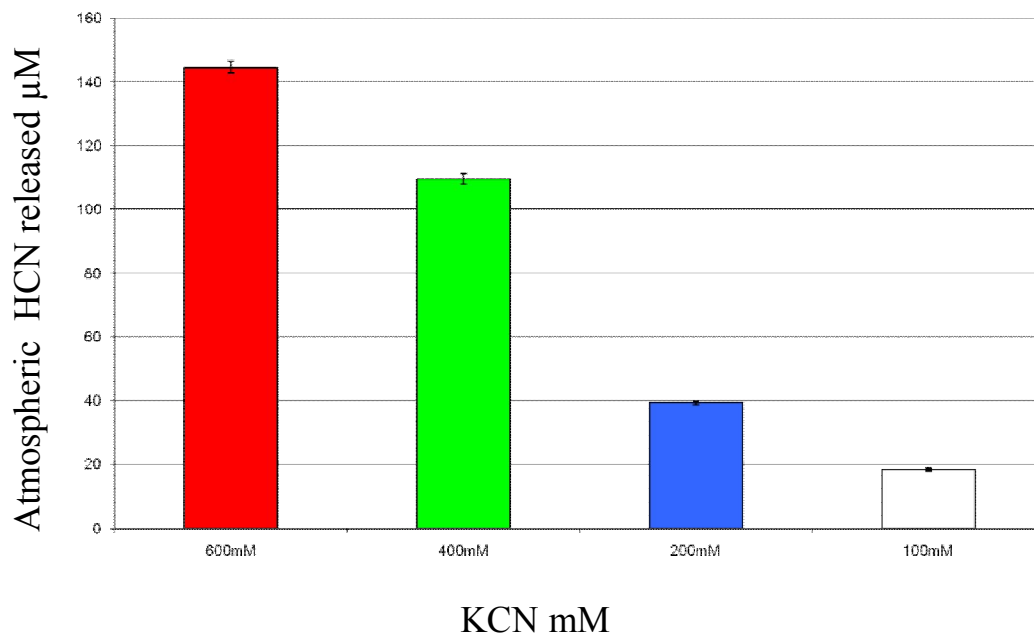
**Figure 4.2** Effect of cyanide treatment on the gene expression of *DR5::GUS* (A) and *ACS6::GUS/GFP* (B) in roots.

### 4.3.2 Determining the cyanide sub-lethal dose to *Arabidopsis* plants

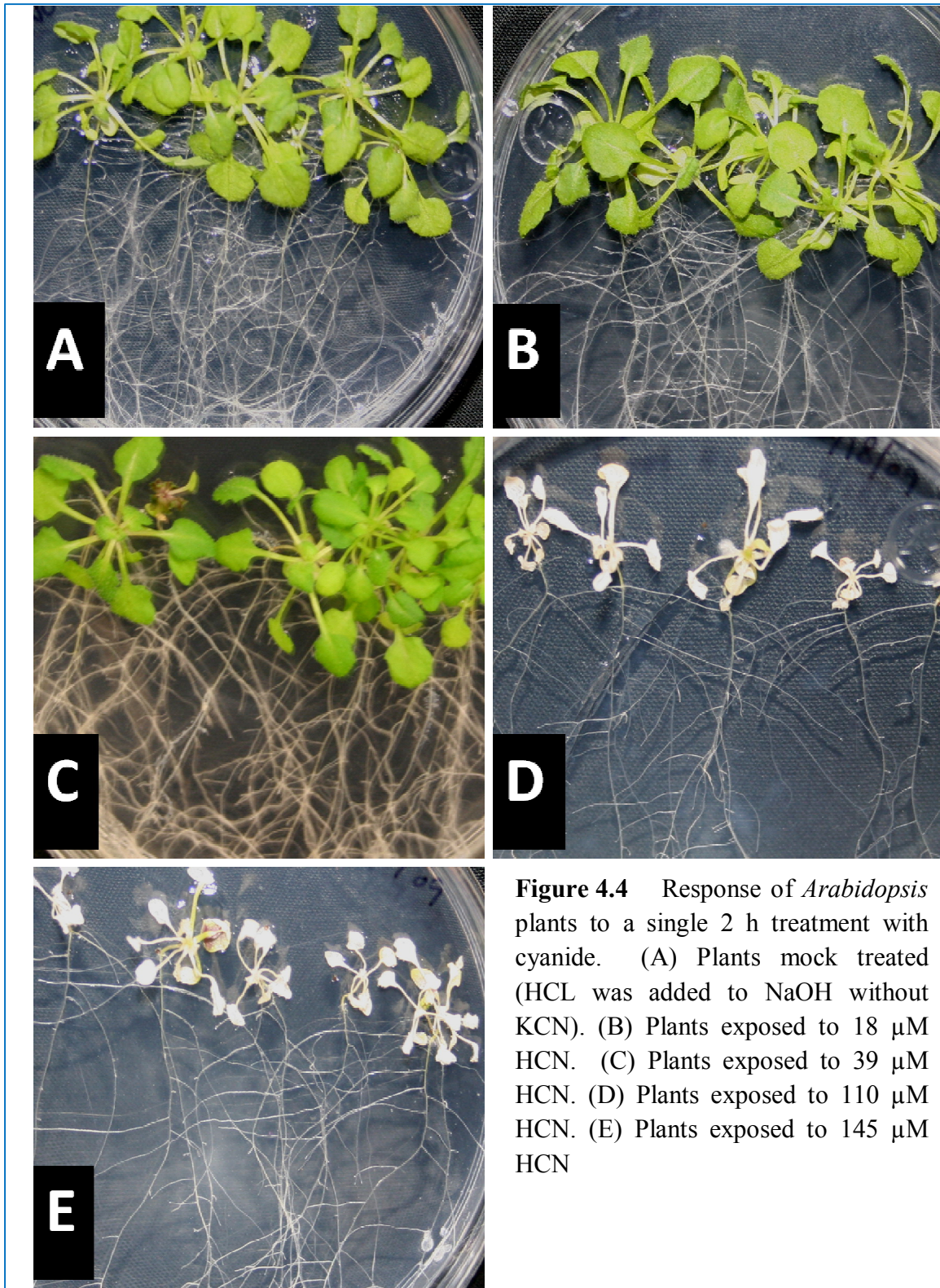
First, we determined the concentration of atmospheric HCN produced over 2 h from the acidification of KCN. Different concentrations of KCN (100, 200, 400, and 600 mM) were acidified inside 10 L dessicator cabinets and the atmospheric HCN produced was captured in NaOH and quantified. From three independent experiments we determined the amount of HCN produced from the degassing of different KCN concentrations. Degassing 100, 200, 400, 600 mM of KCN resulted in 18, 39, 110, 145  $\mu\text{M}$  HCN, respectively, over 2 hours (Figure 4.3) with a coefficient of determination ( $r^2$ ) of 0.979.

Next, we determined the dose of HCN that could be used to induce gene expression without causing any visible alteration to plant growth. Three plates of *Arabidopsis* plants (one month-old) were grown axenically in petri plates (five plants/plate) were challenged with four different concentrations of HCN, 18, 39, 110, 145  $\mu\text{M}$  HCN over 2 hours. To omit the possibility that low concentrations of HCl used to acidify the KCN contributed to the induction of gene expression, control plants were exposed to the same concentration of HCl without the KCN (referred to as mock-treated). After the treatments, the plants were returned back to the growth chamber and plant physical appearance was observed over 10 days to determine the effect of the treatments on plant growth. The results showed that treatment of *Arabidopsis* plants with 18 and 39  $\mu\text{M}$  HCN did not visibly alter plant growth as compared to control plants (Figure 4.4 A & B). The treatment with the two higher HCN concentrations caused bleaching of the leaves after one day of exposure and resulted in plant death after 6 days (Figure 4.4 C & D).





**Figure 4.3** Atmospheric concentrations of HCN produced inside the 10 L dessicator cabinet when KCN was degassed by HCL. Four different concentrations of KCN were used. The HCN is formed when KCN reacts with HCL, which is then captured in a trap containing 5M NaOH. The amount of cyanide in the trap was quantified using a cyanide-ion selective electrode. Data are averages of three independent experiments with three replicates each. Error bars representing the standard errors are shown.



To determine whether the 18 and 39  $\mu\text{M}$  of HCN treatments caused alteration of *Arabidopsis* gene expression, we used quantitative real time PCR (qRT-PCR) to measure any changes of *ACS6* gene expression. Roots and leaves from the treated and mock-treated plants from three individual experiments were harvested and the RNAs were isolated and used for first strand cDNA synthesis. These cDNAs were used in triplicate in SYBR green assays as described in the Materials and Methods section. Data obtained from these experiments were analyzed using  $2^{-\Delta\Delta C_t}$  according to (Livak and Schmittgen, 2001). The *ACS6* gene expression was normalized to the housekeeping gene, actin-2 (*ATACT2*). The results of these experiments are shown in Figure 4.5. The exposure of *Arabidopsis* plants to 18  $\mu\text{M}$  HCN caused 6 and 8-fold changes in *ACS6* gene expression in roots and leaves, respectively (Figure 4.5). Upon exposure to 39  $\mu\text{M}$  HCN, *ACS6* gene expression in roots and leaves was altered 9 and 12-fold, respectively (Figure 4.5).

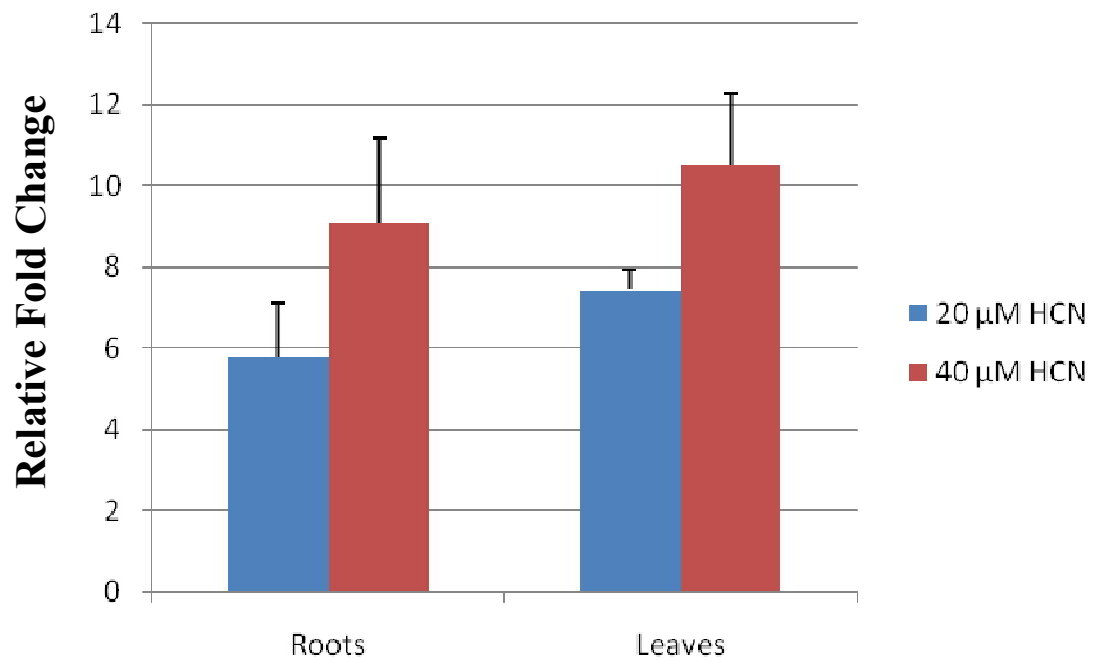
#### **4.3.3 Microarray analysis of gene expression in *Arabidopsis* after cyanide treatment**

To gain a comprehensive understanding of gene expression changes triggered by cyanide treatment in *Arabidopsis* plants, microarray analysis was performed. Experimental set up is similar to that described above. To reduce experimental variations, four replicates were generated for both cyanide-treated and mock-treated control plants. Total RNA was isolated from the root samples and sent for microarray analysis (MOgene, Inc., St. Louis, MO). Four arrays (Agilent, Arabidopsis Array 4) were analyzed using two of the replicates in a dye swap. Statistical analysis of microarray results showed that the expression of a number of genes was significantly

altered ( $p \leq 0.05$ ; FDR=0.2) in response to two hours exposure to cyanide treatment. A total of 2349 genes were significantly differentially regulated during the cyanide treatment. We identified 1004 genes that were up-regulated ( $> 2$ -fold) and 1216 genes that were down-regulated ( $< -2$ ). Using The Gene Ontology (GO) database we found that the largest class of the differentially regulated genes was that with ‘unknown function’. Genes associated with stress responses were twice as likely to be up-regulated (12%) during cyanide treatment as down-regulated (6%) while genes for transport were more likely to be down-regulated (9%) during cyanide treatment than up-regulated (5%).

The clustering of the differentially regulated genes in response to cyanide treatment based on their molecular function (GO terms) resulted in 128 functional groups (Table 4.3). The majority of the differentially regulated genes belong to the following functional categories, “response to reactive oxygen species” (8.7-fold), “response to hypoxia” (7.8-fold), “anaerobic respiration” (5.9-fold), “transcription factors” (5.9-fold), “regulation of defense response” (4.9-fold), “response to cytokinin stimulus” (2.7-fold), “response to cytokinin signaling” (2.6-fold), “metabolic processes” (2.4-fold), and “oxidative response group” (1.8-fold) (Table 4.2).

The analysis of genes associated with nitrogen metabolism revealed differential regulation during HCN treatment (Table 4.2). Genes associated with the CAP pathway (detoxification pathway) were generally up-regulated, including the *nitrilase4* (NIT4), *asparaginase* (ASG) and *glutamate dehydrogeanse* (GDH2) genes.



**Figure 4.5** *ACS6* gene expression analyses in *Arabidopsis* plants in response to different cyanide treatments. RNA was extracted from both root and leaves, cDNAs were synthesized and qPCR was performed using *ACS6* specific primers (DLM & DLN Table 4.1). Data were normalized to the housekeeping gene actin. Data represent averages of three independent experiments and standard errors are represented by error bars.

**Table 4.2** Gene ontology analysis of differentially expressed genes during cyanide treatment.

GO-ID	GO term	Fold change
GO:0000302	response to reactive oxygen species	8.70
GO:0006826	iron ion transport	8.70
GO:0009086	methionine biosynthetic process	8.70
GO:0009218	pyrimidine ribonucleotide metabolic process	8.70
GO:0045298	tubulin complex	8.16
GO:0001666	response to hypoxia	7.83
GO:0005985	sucrose metabolic process	7.83
GO:0006995	cellular response to nitrogen starvation	7.83
GO:0009612	response to mechanical stimulus	7.83
GO:0010105	negative regulation of ethylene mediated signaling pathway	7.46
GO:0004673	protein histidine kinase activity	7.12
GO:0010380	regulation of chlorophyll biosynthetic process	6.53
GO:0043069	negative regulation of programmed cell death	6.53
GO:0009061	anaerobic respiration	5.93
GO:0009638	Phototropism	5.80
GO:0010089	xylem histogenesis	5.80
GO:0008266	poly(U) RNA binding	5.59
GO:0002213	defense response to insect	5.59
GO:0006879	cellular iron ion homeostasis	5.59
GO:0008134	transcription factor binding	5.59
GO:0009765	photosynthesis, light harvesting	5.59
GO:0031012	extracellular matrix	5.59
GO:0008375	acetylglucosaminyltransferase activity	5.44
GO:0006914	Autophagy	5.22
GO:0009306	protein secretion	5.22
GO:0010075	regulation of meristem growth	5.22
GO:0016772	transferase activity, transferring phosphorus-containing groups	5.22
GO:0046914	transition metal ion binding	5.22
GO:0051537	2 iron, 2 sulfur cluster binding	5.22
GO:0009630	Gravitropism	5.02
GO:0007155	cell adhesion	4.90
GO:0009927	histidine phosphotransfer kinase activity	4.90
GO:0010149	Senescence	4.90
GO:0019253	reductive pentose-phosphate cycle	4.90
GO:0031347	regulation of defense response	4.90
GO:0016207	4-coumarate-CoA ligase activity	4.75
GO:0048767	root hair elongation	4.75
GO:0009825	multidimensional cell growth	4.66
GO:0009742	brassinosteroid mediated signaling	4.57



GO-ID	GO term	Fold change
GO:0009863	salicylic acid mediated signaling pathway	4.35
GO:0010286	heat acclimation	4.35
GO:0009698	phenylpropanoid metabolic process	4.35
GO:0009718	anthocyanin biosynthetic process	4.35
GO:0010114	response to red light	4.23
GO:0000325	plant-type vacuole	4.18
GO:0009626	plant-type hypersensitive response	4.18
GO:0005986	sucrose biosynthetic process	4.08
GO:0008171	O-methyltransferase activity	4.08
GO:0005774	vacuolar membrane	3.84
GO:0009414	response to water deprivation	3.82
GO:0009637	response to blue light	3.73
GO:0005768	Endosome	3.73
GO:0018119	peptidyl-cysteine S-nitrosylation	3.73
GO:0046658	anchored to plasma membrane	3.56
GO:0016051	carbohydrate biosynthetic process	3.50
GO:0006825	copper ion transport	3.48
GO:0009741	response to brassinosteroid stimulus	3.48
GO:0048573	photoperiodism, flowering	3.26
GO:0016126	sterol biosynthetic process	3.26
GO:0007568	Aging	3.18
GO:0010200	response to chitin	3.18
GO:0005198	structural molecule activity	3.14
GO:0016168	chlorophyll binding	3.01
GO:0010319	Stromule	2.95
GO:0006970	response to osmotic stress	2.93
GO:0031977	thylakoid lumen	2.84
GO:0030154	cell differentiation	2.84
GO:0005783	endoplasmic reticulum	2.74
GO:0009735	response to cytokinin stimulus	2.73
GO:0009624	response to nematode	2.72
GO:0009607	response to biotic stimulus	2.70
GO:0046983	protein dimerization activity	2.69
GO:0004712	protein serine/threonine/tyrosine kinase activity	2.61
GO:0009736	cytokinin mediated signaling	2.61
GO:0010218	response to far red light	2.61
GO:0009809	lignin biosynthetic process	2.61
GO:0046686	response to cadmium ion	2.59
GO:0009744	response to sucrose stimulus	2.54
GO:0015250	water channel activity	2.54
GO:0008219	cell death	2.53
GO:0009644	response to high light intensity	2.53

<b>GO-ID</b>	<b>GO term</b>	<b>Fold change</b>
GO:0009651	response to salt stress	2.44
GO:0009407	toxin catabolic process	2.40
GO:0044237	cellular metabolic process	2.40
GO:0006334	nucleosome assembly	2.37
GO:0009416	response to light stimulus	2.33
GO:0050832	defense response to fungus	2.30
GO:0009617	response to bacterium	2.29
GO:0009751	response to salicylic acid stimulus	2.29
GO:0000786	Nucleosome	2.28
GO:0035091	phosphoinositide binding	2.28
GO:0005618	cell wall	2.25
GO:0009409	response to cold	2.19
GO:0009753	response to jasmonic acid stimulus	2.18
GO:0005576	extracellular region	2.18
GO:0042742	defense response to bacterium	2.16
GO:0009408	response to heat	2.15
GO:0005886	plasma membrane	2.14
GO:0005773	Vacuole	2.06
GO:0009723	response to ethylene stimulus	2.06
GO:0009826	unidimensional cell growth	2.04
GO:0009611	response to wounding	2.03
GO:0005829	Cytosol	2.00
GO:0009505	plant-type cell wall	1.98
GO:0048046	Apoplast	1.92
GO:0006629	lipid metabolic process	1.88
GO:0009941	chloroplast envelope	1.86
GO:0009737	response to abscisic acid stimulus	1.84
GO:0006979	response to oxidative stress	1.83
GO:0016787	hydrolase activity	1.77
GO:0004722	protein serine/threonine phosphatase activity	1.75
GO:0009579	Thylakoid	1.74
GO:0005507	copper ion binding	1.72
GO:0005777	Peroxisome	1.72
GO:0022626	cytosolic ribosome	1.70
GO:0016020	Membrane	1.70
GO:0006810	Transport	1.61
GO:0009570	chloroplast stroma	1.59
GO:0016757	transferase activity, transferring glycosyl groups	1.57
GO:0009535	chloroplast thylakoid membrane	1.56
GO:0005737	Cytoplasm	1.55
GO:0005215	transporter activity	1.47
GO:0005730	Nucleolus	1.47



<b>GO-ID</b>	<b>GO term</b>	<b>Fold change</b>
GO:0005488	Binding	1.35
GO:0005739	Mitochondrion	1.33
GO:0009507	Chloroplast	1.32
GO:0003824	catalytic activity	1.27
GO:0005634	Nucleus	1.24

#### 4.3.4 Validation of microarray data by qPCR

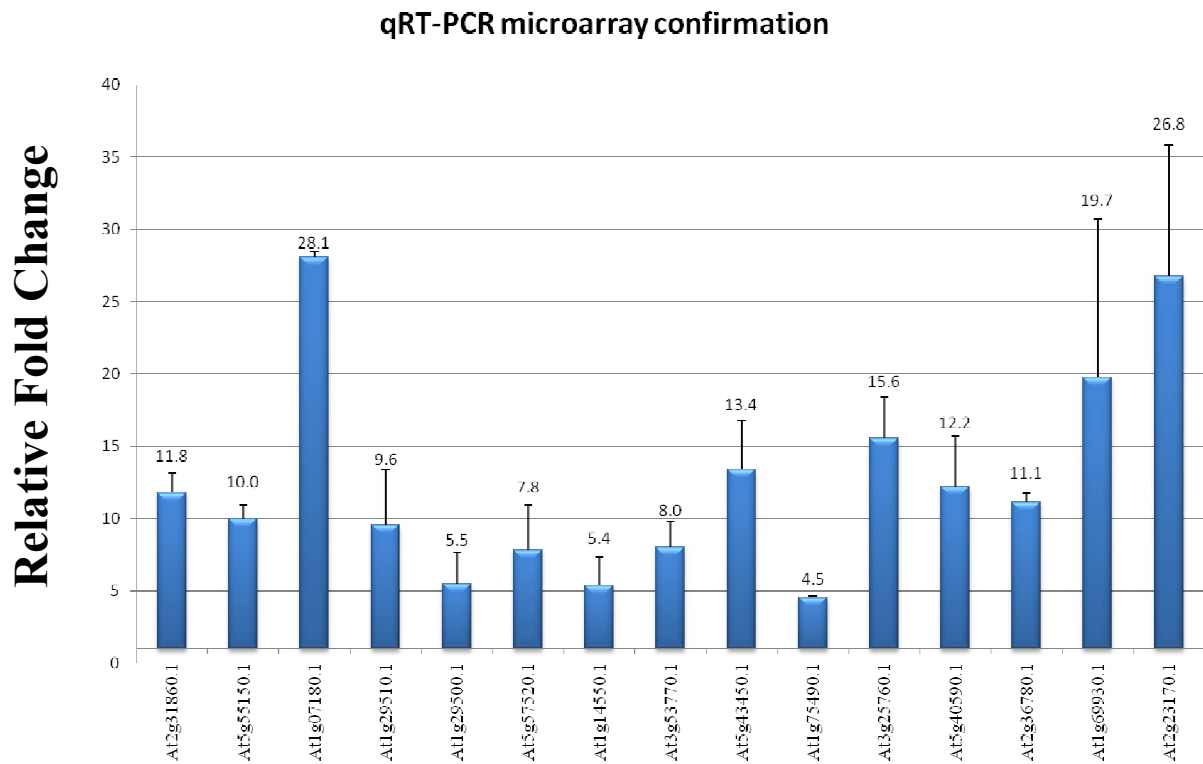
Subsequent to analyzing the microarray data, a set of 15 genes was chosen for validation by quantitative real time PCR based on their high fold changes, p-value, and possible response to cyanide treatment. In addition, eight other genes associated with cyanide detoxification were also selected for validation. The list of the genes selected (total 23 genes) for qPCR validation with their primer sequences is given in Table 4.1. The microarray results for these 23 genes along with their AGI identification, p-values and the GO terms are given in Table 4.3.

In order to determine the reliability and accuracy of the microarray data, three different cyanide-treatment experiments independent from those used for the microarray analysis were used for qPCR validation. The quantification of gene expression was performed using a relative quantification method ( $2^{-\Delta\Delta C_t}$ ). The qPCR quantitative results for the set of 15 genes (Figure 4.6) without exceptions were in agreement with the microarray results and reinforced our microarray findings. On the other hand, qPCR analysis for genes associated with cyanide detoxification (CAP;  $\beta$ -cyanoalanine pathway) (Figure 4.6) were in agreement with the microarray results (Table 4.3) except for one gene. A gene encoding a nitrate transporter 1:2 (AT1G69850.1), which was identified as up-regulated by microarray analysis, was shown to be down-regulated by qPCR (Figure 4.7). Collectively, the results from all qPCR analysis clearly confirm the microarray results.

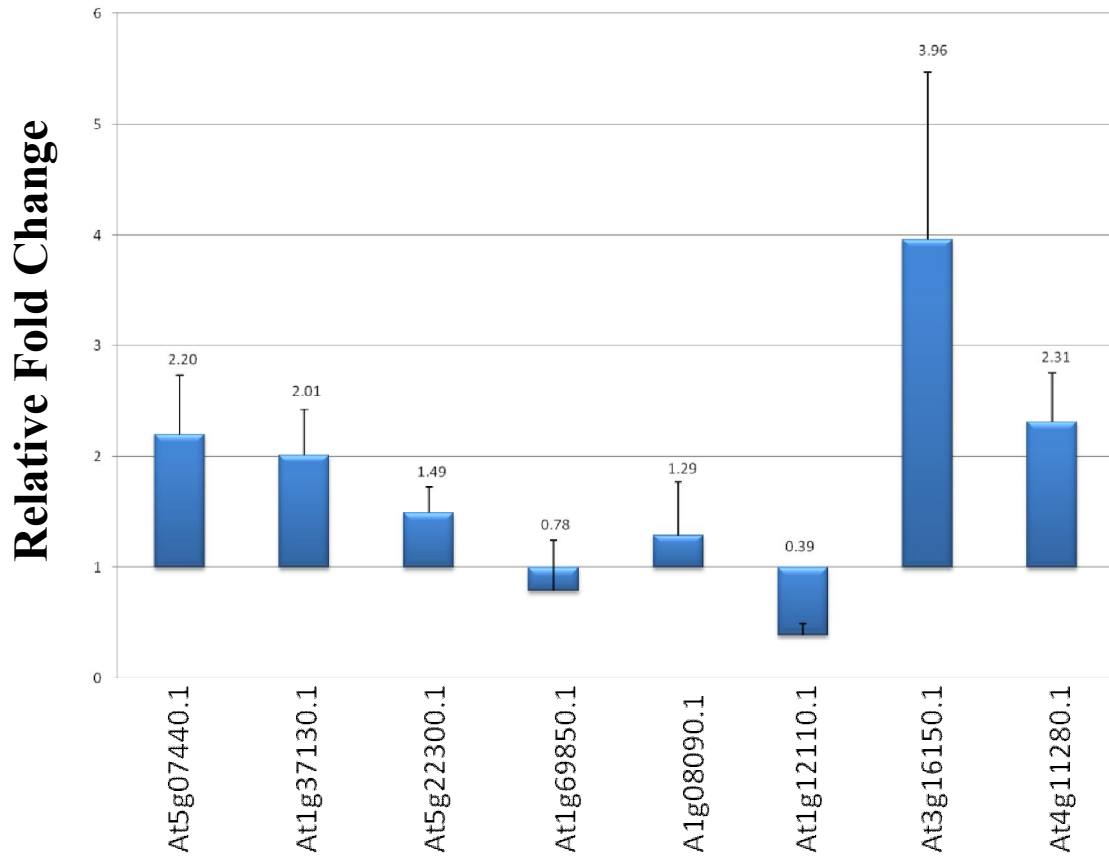
**Table 4.3** Genes used for the validation of microarray analysis. The description and Go term refer to automatic Arabidopsis annotations according to AGI number from The Institute for Genomic Research and Gene Ontology Database.

Gene name	AGI name	Description	Go term	Microarray	qPCR
AT2G31860	AT2G31860.1	Putative poly(ADP-ribose) glycohydrolase	Unclassified proteins	50.75	11.8
AT5G55150	AT5G55150.1	F-box family protein similar to unknown protein	Molecular function	51.00	10
ATNDI1/NDA1	AT1G07180.1	Pyridine nucleotide-disulphide oxidoreductase family protein con	NADH dehydrogenase activity	50.50	28
SAUR68	AT1G29510.1	Auxin-responsive protein, putative similar to auxin-induced protein	Molecular function	40.50	9.6
AT1G29500	AT1G29500.1	Auxin-responsive protein, putative similar to auxin-induced protein	Molecular function	39.25	5.5
ZFP2	AT5G57520.1	Zinc finger (C2H2 type) family protein (ZFP2)	Nuclear acid binding	35.25	7.8
AT1G14550	AT1G14550.1	Anionic peroxidase, putative similar to anionic peroxidase GI:17	Peroxidase activity	34.25	5.4
AT3G53770	AT3G53770.1	Late embryogenesis abundant protein-related / LEA protein	Molecular function	34.75	8
AT5G43450	AT5G43450.1	2-oxoglutarate-dependent dioxygenase, putative similar to 2A6	Biological process	34.00	13.4
AT1G75490	AT1G75490.1	DRE-binding transcription factor, putative similar to DREB2A GB	DNA binding	18.13	4.5
AOC1	AT3G25760.1	Early-responsive to dehydration stress protein (ERD12)	Response to desiccation	37.75	15.6
AT5G40590	AT5G40590.1	DC1 domain-containing protein predicted protein	Biological process	27.75	12.2
AT2G36780	AT2G36780.1	UDP-glucuronosyl/UDP-glucosyl transferase family protein	Biological process	21.75	11.1
ATGSTU11	AT1G69930.1	Glutathione S-transferase	Glutathione transferase activity	45.75	19.7
GH3.3	AT2G23170.1	Auxin-responsive GH3 family protein	Auxin response	44.00	26.8
Actin2	AT3G18780.1	Actine gene; structural constituent of cytoskeleton	structural constituent of cytoskeleton	0.25	
<b>Genes involved in nitrogen and ethylene</b>					
GDH2	AT5G07440.1	Glutamate dehydrogenase 2, oxidoreductase	Glutamate dehydrogenase activity	8.33	2.2
NIA2	AT1G37130.1	Nitrate reductase 2	Nitric oxide biosynthetic process	9.06	2,01
NIT4	AT5G22300.1	Nitralase 4	Nitrilase activity	10.75	1.49
ATNRT1:2	AT1G69850.1	Nitrate transporter 1:2 calcium ion binding	Transporter activity	8.25	0.78
ATNRT2:1	AT1G08090.1	Arabidopsis thaliana high affinity nitrate transporter	Response to nitrate	-11.75	1.29
NRT1.1	AT1G12110.1	Nitrate transporter 1:1	Transporter activity	-5.00	0.39
AT3G16150	AT3G16150.1	L-asparaginase, putative / L-asparagine amidohydrolase	Asparaginase activity	24	3.96
ACS6	AT4G11280.1	1-aminocyclopropane-1-carboxylic acid (ACC) synthase 6	Ethylene biosynthetic process	12	2.3

Positive values represent genes that were up-regulated and negative values represent genes that were down-regulated during cyanide treatment. Microarray, represent fold change values estimated without data normalization. qPCR represent fold change normalized to control.



**Figure 4.6** Validation of microarray results for 15 genes with qPCR. RNAs were extracted from root, cDNAs were synthesized and qPCR was performed using gene specific primers (Table 4.1). Data were normalized to the housekeeping gene actin. Data represent averages of three experiments independent from those used for conducting the microarray. Standard errors are represented by error bars.

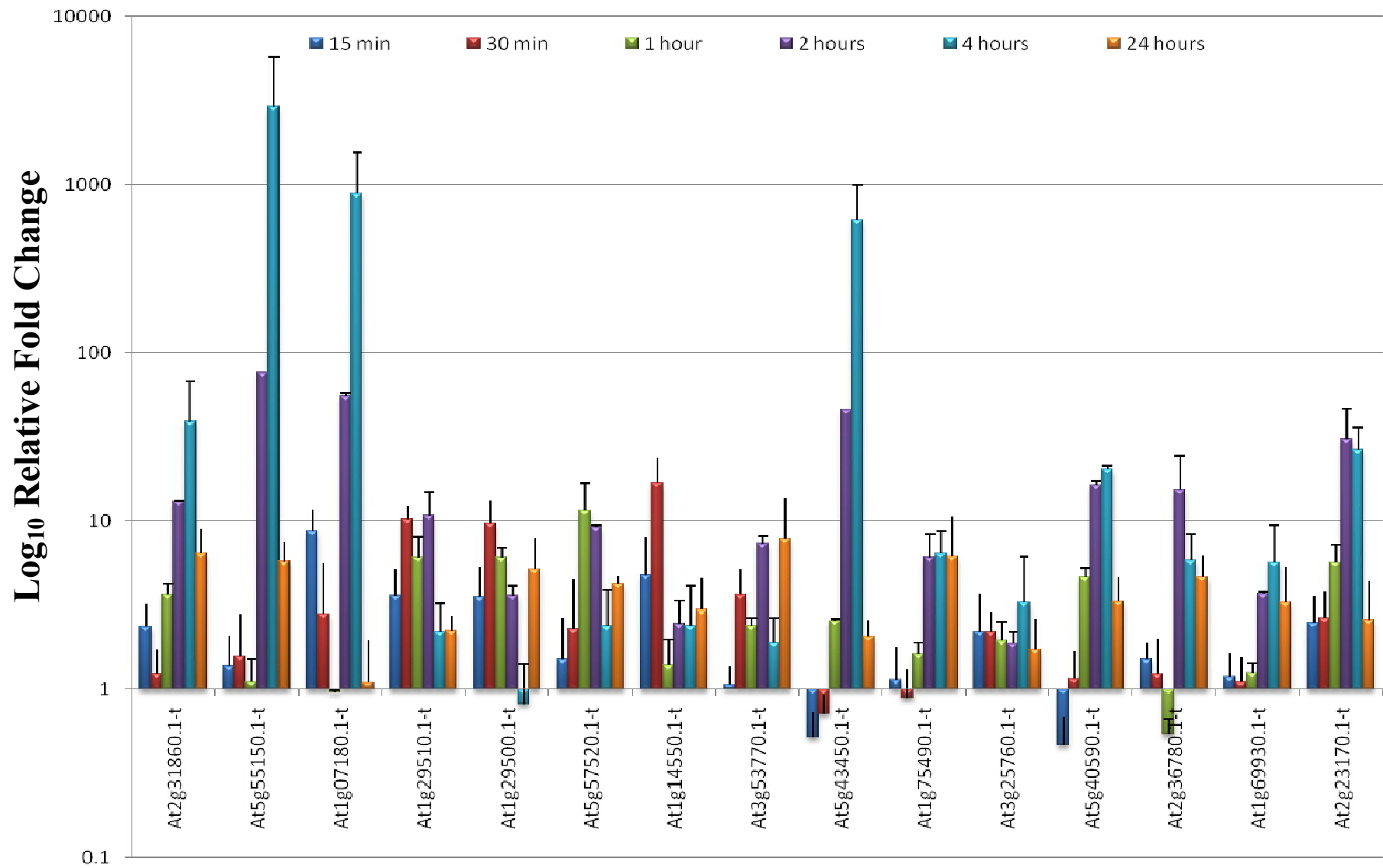


**Figure 4.7** Validation of microarray results for plant cyanide detoxification genes with qPCR. RNAs were extracted from root, cDNAs were synthesized and qPCR was performed using gene specific primers (Table 4.1). Data were normalized to the housekeeping gene actin. Data represent averages of three experiments independent from those used for conducting the microarray. Standard errors are represented by error bars.

#### 4.3.5 Time-course analysis of gene expression in response to cyanide treatment

To identify any correlation between transcriptional responses and cyanide treatments, a time-course series of experiments was carried out. The expression of a set of 15 genes that were among the highest up-regulated genes in the microarray experiment (Table 4.3) was monitored over time in response to a single cyanide treatment. The time points used were 15 min, 30 min, 1 h, 2 h, 2 h + 2 h recovery (referred to as 4 h) and 2 h + 22 h recovery (referred to as 24 h). The quantitative qPCR analysis showed that out of the 15 genes examined, only eight genes responses peaked following the 15 min cyanide exposure (Figure 4.8). Three of these eight genes continued the increased expression after 30 min of cyanide exposure (Figure 4.8). Aside from these eight genes, expression of two other genes peaked after 30 min exposure (Figure 4.8). After 2 hours of cyanide exposure, all 15 genes were up-regulated (Figure 4.8) similar to that observed in the microarray and qPCR results.

Furthermore, we were interested to determine if the expression of these 15 genes would change if given time to recover after 2 hours cyanide treatment ended. After giving 2 hours recovery period, five genes continued to increase their level of expression, two genes maintained the expression level, and eight genes showed decreased levels compared to that of 2 hours exposure with no recovery time (Figure 4.8). To take it a step further, we provided a 22 h recovery period after the 2 hours exposure. Only two genes continued to increase their level of expression, four genes maintained the increased expression level, and nine genes showed decreased levels compared to that of 2 hours exposure with 2 hours recovery time (Figure 4.8).



**Figure 4.8** Time course and gene expression response to cyanide treatments. *Arabidopsis* plants were challenged with a single dose of 20  $\mu$ M HCN and root samples were collected at 15 min, 30 min, 1 h, 2 h. The 4 and 24 h time points represent 2 h exposures but root samples were collected after additional 4 and 24 hours, respectively. RNAs were extracted from root, cDNAs were synthesized and qPCR was performed using gene specific primers (Table 4.1). Data were normalized to the housekeeping gene actin. Data represent averages of three experiments and standard errors are represented by error bars



#### 4.3.6 Evaluation of *Arabidopsis* biomarkers in response to bacterial cyanide

The influence of cyanide produced by *Pseudomonas* sp. strain 15G2 on the expression of the 23 genes previously tested using HCN (produced from the acidification of KCN) was determined under both laboratory and soil conditions.

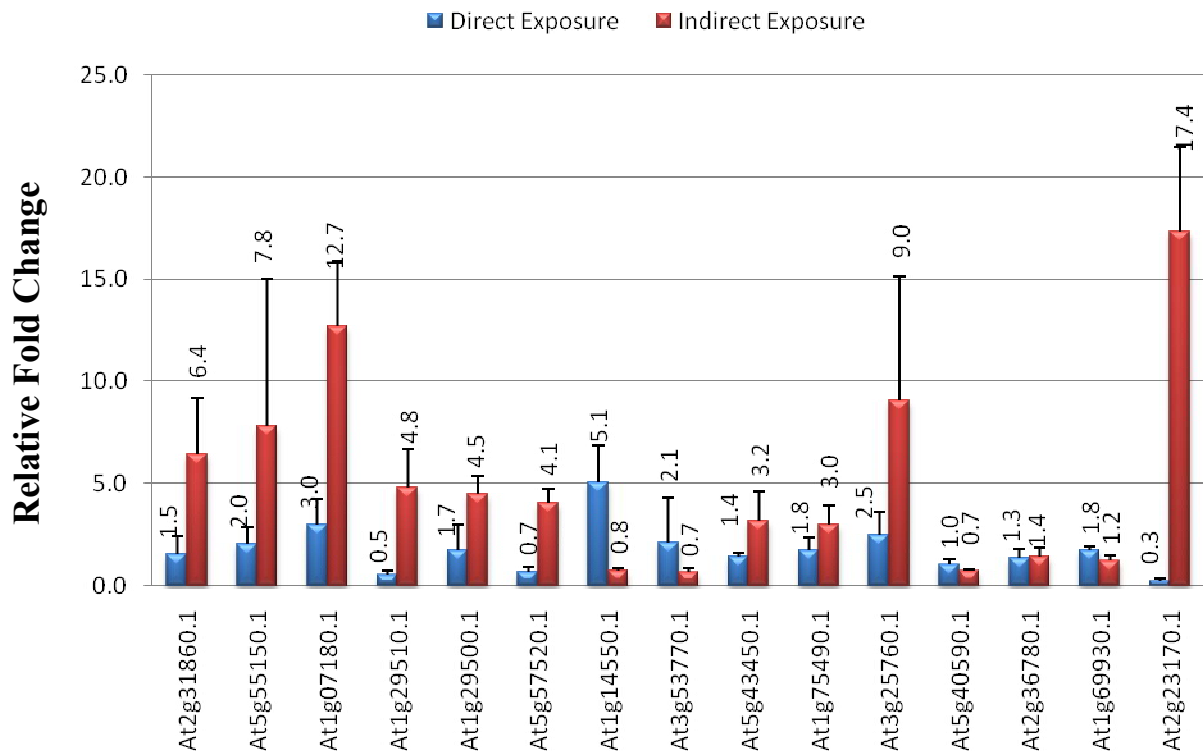
***In vitro evaluation.*** Two sets of experiments were performed. In the first set of experiments, strains WT-15G2 and 15G2-*AhcnABC* were individually directly applied along the root systems of one month old *Arabidopsis* plants growing on Gamborg's B5 media in petri plates. The plates were placed in a growth chamber (22 °C) for 48 hours. In the second set of experiments, to eliminate a possibility that bacteria itself could alter the expression of selected genes, LB plates inoculated with the bacterial strains were allowed to share air for 24 hours with one month old *Arabidopsis* plants growing on separate plates. From both experiments, root samples were harvested, RNAs were isolated, and cDNAs were generated and used for qPCR analysis. The gene expression of *Arabidopsis* plants exposed or inoculated with WT strain 15G2 were normalized to that of plants exposed to 15G2-*AhcnABC*. Among the 15 genes tested, ten genes showed up-regulation when *Arabidopsis* plants were indirectly exposed to cyanide produced by 15G2 (Figure 4.9). However, only two genes were up-regulated when *Arabidopsis* plants were directly inoculated with strain 15G2 (Figure 4.9). These two genes were up-regulated in both plants inoculated with WT strain 15G2 and 15G2-*AhcnABC* indicating that the up-regulation was due to the bacterial presence and not because of cyanide exposure.

RNAs from the above two sets of experiments were also used to analyze the expression of genes associated with the cyanide detoxification CAP pathway. The

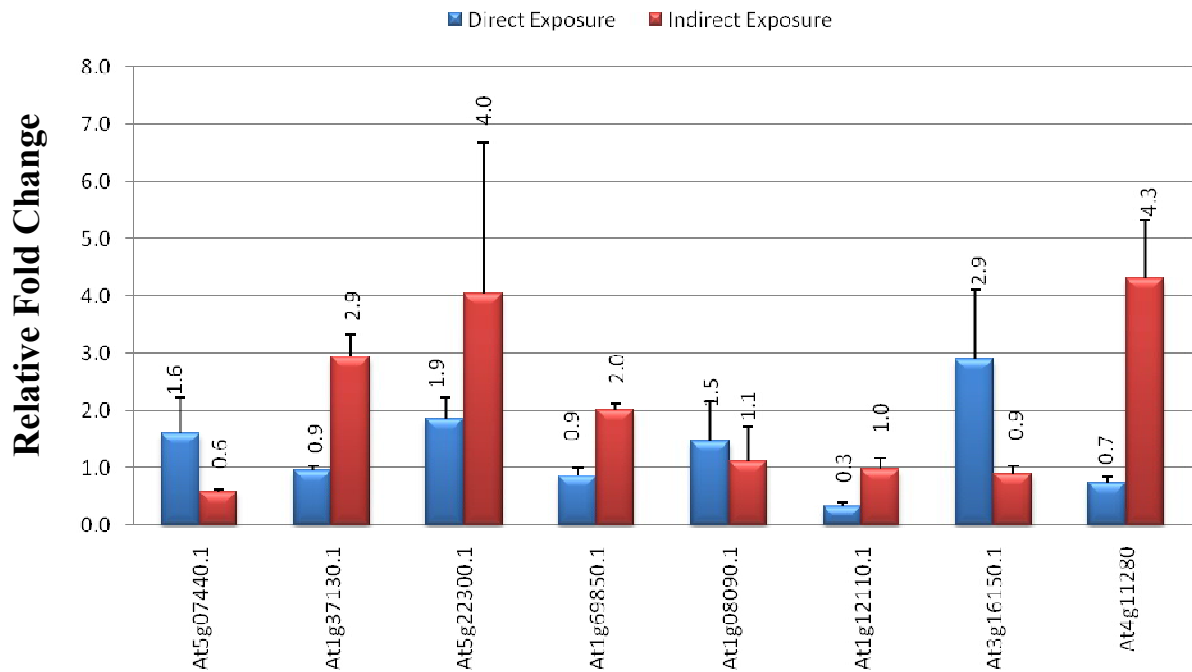
quantitative qPCR results showed that among all up-regulated genes, the expression of two genes was due to bacterial presence (up-regulated in plants inoculated with WT strain 15G2 and 15G2-*ΔhcnABC*) and five other genes were up-regulated due to cyanide exposure (up-regulated only in plants sharing air with WT strain 15G2) (Figure 4.10).

***In vivo evaluation.*** The differentially regulated genes that were identified using the microarray and qPCR experiments were used to evaluate the effect of cyanide produced by strain 15G2 on *Arabidopsis* gene expression under soil conditions. Three week old *Arabidopsis* plants growing in soil were inoculated separately by WT strain 15G2 and 15G2-*ΔhcnABC*. After one week, the roots were harvested, RNAs extracted, and examined using qPCR. To examine the role bacterial produced HCN has on gene expression, we examined gene expression during treatments with 15G2 and the reduced cyanide producing strain 15G2- *ΔhcnABC*. Three different biological replicates for each treatment were examined. The data were analyzed using a relative quantification method ( $2^{-\Delta\Delta C_t}$ ) and a 2 fold expression difference cut-off. Our results showed that eight out of the 15 genes that were tested responded to bacterial cyanide under soil conditions (Figure 4.11). These genes can be grouped in three categories: (i) highly up-regulated, which include At5g55150 (140-fold) and At5g43450 (72-fold); (ii) Moderately up-regulated, which include At2g31860 (~ 28-fold), At1g07180 (~ 25-fold), and At3g25760 (~ 22-fold); (iii) and slightly up-regulated, which include At1g75490 (3-fold), At1g69930 (4.5-fold), and At2g23170 (3-fold) (Figure 4.11). The data from these experiments clearly suggest that HCN is being produced at the soil level by 15G2 and plants can sense this cyanide and alter their gene expression.

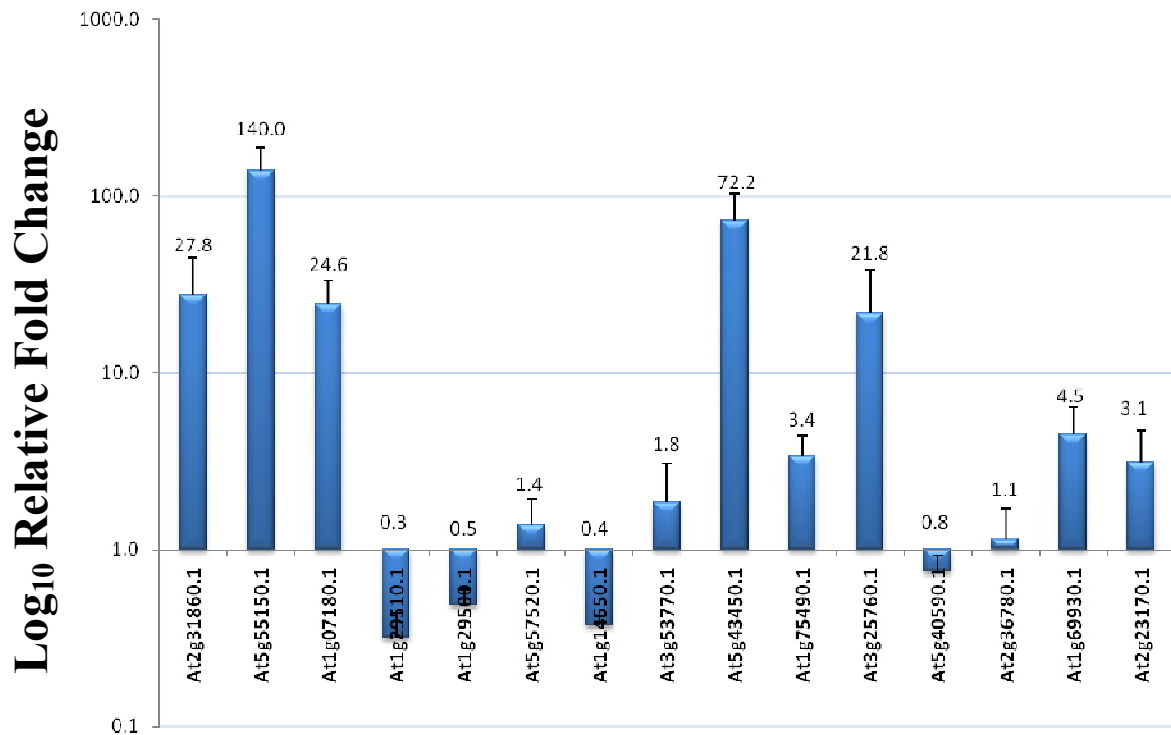
Next, we questioned whether plants can cope with the cyanide produced by bacteria in soil. RNAs from the above experiments were used to analyze the expression of genes associated with the cyanide detoxification CAP pathway. The quantitative qPCR results were comparable to that obtained before from microarray and qPCR *in vitro* experiments (Figure 4.12). These results indicated that bacterial cyanide can be absorbed, detoxified and incorporated in the nitrogen pool.



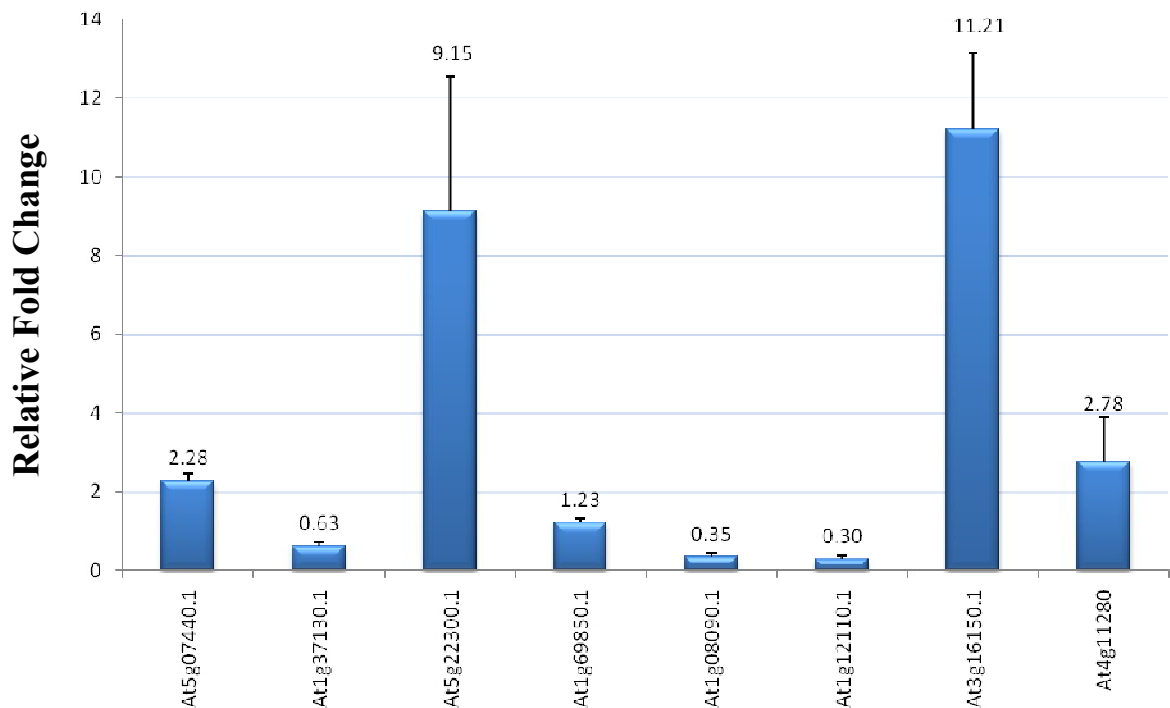
**Figure 4.9** Evaluation of *Arabidopsis* gene expression in response to direct and indirect exposure to strain 15G2 *in vivo* using qPCR. For the direct exposure, the *Arabidopsis* plants were inoculated along the root system and samples were collected after 2 days. For the indirect exposure, plants and bacteria were allowed to share the same air for 1 day before collecting samples. RNAs were extracted from root, cDNAs were synthesized and qPCR was performed using gene specific primers (Table 4.1). Plants inoculated or exposed to 15G2- $\Delta hcnAB$  (defective in HCN production) were used as a calibrator to eliminate those biotic effects on gene expression and a 2 fold expression difference cut-off was used. Data represent averages of three and standard errors are represented by error bars.



**Figure 4.10** Evaluation of *Arabidopsis* cyanide-detoxification genes in response to direct and indirect exposure to strain 15G2 *in vivo* using qPCR. For the direct exposure, the *Arabidopsis* plants were inoculated along the root system and samples were collected after 2 days. For the indirect exposure, plants and bacteria were allowed to share the same air for 1 day before collecting samples. RNAs were extracted from root, cDNAs were synthesized and qPCR was performed using gene specific primers (Table 4.1). Plants inoculated or exposed to 15G2-*ΔhcnAB* (defective in HCN production) were used as calibrators to eliminate those biotic effects that could contribute to gene expression and a 2 fold expression difference cut-off was used. Data represent averages of three and standard errors are represented by error bars.



**Figure 4.11** Evaluation of *Arabidopsis* gene expression in soil in response to the presence of strain 15G2 using qPCR. The *Arabidopsis* plants were inoculated with strain 15G2 ( $10^4 - 10^6$  cfu/g of soil) and root samples were collected after 7-days. RNAs were extracted from root, cDNAs were synthesized and qPCR was performed using gene specific primers (Table 4.1). Plants inoculated with 15G2-*AhcnAB* (defective in HCN production) were used as a calibrator to eliminate those biotic effects that could contribute to gene expression. Data represent averages of three and standard errors are represented by error bars.



**Figure 4.12** Evaluation of *Arabidopsis* cyanide-detoxification gene expression in soil in response to the presence of strain 15G2 using qPCR. The *Arabidopsis* plants growing were inoculated with strain 15G2 ( $10^4 - 10^6$  cfu/g of soil) and root samples were collected after 7-days. RNAs were extracted from root, cDNAs were synthesized and qPCR was performed using gene specific primers (Table 4.1). Plants inoculated with 15G2-*ΔhcnAB* (defective in HCN production) were used as a calibrator to eliminate those biotic effects that could contribute to gene expression. Data represent averages of three and standard errors are represented by error bars.

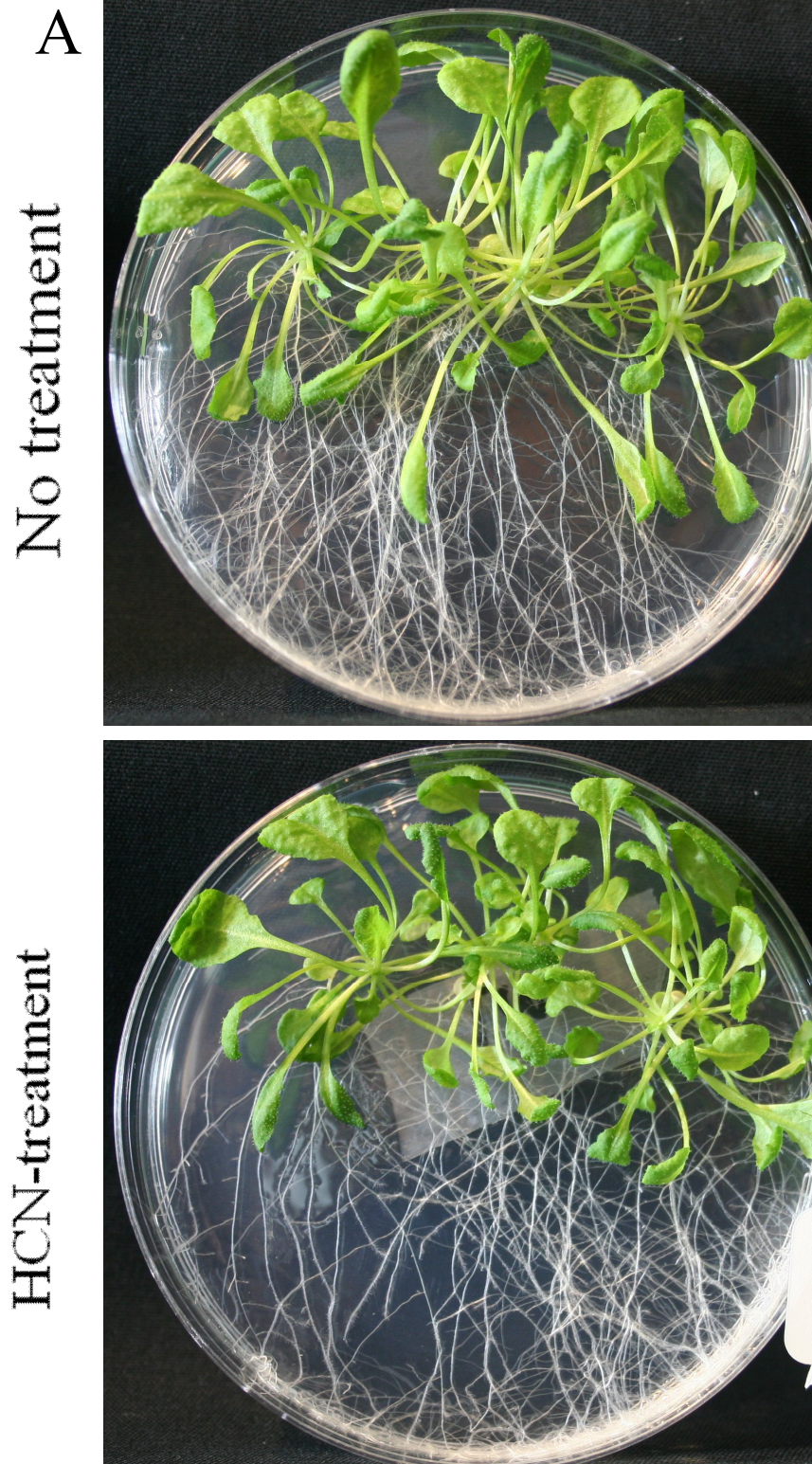
#### 4.3.7 Effects of cyanide treatments on knockout mutants phenotypes

In order to further determine if genes that were up-regulated upon cyanide treatment have any effect on plant growth and development, we searched the Salk Institute Insertional Mutation Database to find any T-DNA insertion mutants (Alonso et al., 2003) for those genes that were highly up-regulated. Unfortunately, we only found six mutants for the genes tested in the above experiments. However, we were able to find ten more mutants that correspond to the top 100 up-regulated genes in the microarray results. The mutant (Salk) -lines are listed in the Materials and Methods section. Most of these mutants showed phenotypic differences before cyanide treatment compared to WT plants. Among these phenotypic differences is the small overall size of the plants. Three week old *Arabidopsis* mutant plants received a single 2 hour exposure to 18  $\mu$ M HCN and were placed back in the growth chamber for 10 days. Five out of the 16 mutants that were tested showed visible phenotypic changes on roots or shoots or both. Three mutations affected the overall plant size. Leaves exhibited a ‘cupping’ phenotype (indicative of stress), and had significantly shorter lateral roots when compared to the phenotype of untreated plants (Figure 4.13 B&E&F). These mutations are in At4g35770 (Salk 020571) that encodes a senescence-associated gene, At5g57520 (Salk 007343) that encodes a zinc finger protein, and At3g61440 (*sas*; 3:1) that encodes a cysteine synthase isomer. The other two mutations had distinct phenotypes. The mutation in At2g31860 (Salk 124967) that encodes for pseudogene (ADP-ribose glycohydrolase) significantly affected both the sizes of the leaves and root mass (Figure 4.13D). This gene was highly up-regulated in both the microarray analysis and in all qPCR experiments done in this study. The other mutant was in At1g15040 (Salk



031983) that encodes for glutamine amidotransferase. This mutant caused primary root inhibition and increased the length of the lateral roots compared to untreated plants.

WT





Salk-020571

B

No treatment



HCN-treatment



Salk-031983

No treatment C



HcN-treatment





Salk-124967

D  
No treatment



HCN-treatment





Salk-007343

No treatment E



HCN-treatment





Sas; 3:1

No treatment



HCN-treatment



F

**Figure 4.13** Phenotypic characterization of *Arabidopsis* plants carrying mutations in genes that were up-regulated during cyanide treatment. The mutants (SALK lines) received a single 2 h exposure to 20 M HCN and their phenotypes were visually monitored over 10 days. Wild type *Arabidopsis* plants were used as a control. For details about these SALK lines and their phenotypes, see text.

(A) Wild type *Arabidopsis* (B) SALK-020571C (C) SALK-031983C (D) SALK-124967C (E) SALK-007343C (F) Sas;3:1

#### 4.4 Discussion

In an attempt to find plant biomarkers for cyanide exposure, we evaluated the direct and indirect effects of strain 15G2 on both *ACS6::GUS/GFP* and *DR5::GUS* gene expression. Our results indicated that neither *ACS6* nor *DR5::GUS* can be used as a biomarker for cyanide treatment. The *ACS6* gene is shown to be a multi-responsive gene, as its gene expression may be induced by touch, wounding, and by treatment with auxin, LiCl, NaCl, CuCl<sub>2</sub>, cycloheximide (CHX), aminooxyacetic acid (AOA) and ethylene (Arteca and Arteca, 1999). This may explain why we could not differentiate between *ACS6::GUS/GFP* gene expression among treatments, because touching or wounding may occur when handling the seedlings during the experiments. Furthermore, the GUS staining solution contains 0.5 mM potassium ferricyanide, which could account for the GUS activity noticed in both *ACS6::GUS/GFP* and *DR5::GUS* transgenic plants inoculated with either WT strain 15G2 or *15G2-ΔhcnABC*. All in all, we decided to use a microarray-based approach for a genome-wide survey to identify genes that are highly responsive to cyanide presence.

To our knowledge, this is the first study comprising gene expression analysis of *Arabidopsis* exposed to cyanide treatment. Using the Affymetrix Arabidopsis Genome Array chip that contains approximately 24,000 genes, we were able to compile a list of genes whose expression was altered in response to a single two hour exposure to 18 μM HCN. These conditions were chosen for conducting the microarray experiment because under these conditions plant growth and appearance were not affected and resulted in *ACS6* gene expression. Approximately 9.8% of the 24,000 *Arabidopsis* genes represented on the Affymetrix chip displayed significant changes in mRNA levels in



response to cyanide treatment. The strongest significantly up-regulated gene expression observed was between 52 and 67-fold for genes encoding unknown proteins (AT5G24640.1, AT4G12735.1, AT3G54530.1, AT2G41730.1, and AT5G55150.1). In addition, several stress response genes with known functions were also highly up-regulated. For example, the gene encoding glutathione-S-transferase (At1g69930) has a 45-fold increase in the mRNA levels compared to control plants. This gene belongs to the glutathione transferase family, which is known to have roles in herbicide detoxification (Marrs, 1996), stress signaling (Loyall et al., 2000) and detoxification of organic hydroperoxides that form during oxidative stress (Edwards et al., 2000). In mammals, the glutathione transferase genes contribute to resistance to cyanogens and products of oxidative stress (Hayes and Pulford, 1995). Furthermore, genes that encode transcription factors such as WRKY that are known to be involved in biotic and abiotic stress responses (Wei et al., 2008) were also up regulated.

The clustering analysis of the differentially expressed genes clearly indicate that several molecular processes were regulated by cyanide treatment. The coordination between these molecular processes requires a complex regulation system. For example, genes encoding several transcription factors were highly regulated in response to cyanide treatment. In general, the majority of the genes that were highly differentially regulated during the cyanide treatment were those that respond to reactive oxygen species (8.7-fold) and hypoxia (7.8-fold). The root apical cells have a high demand for oxygen and when the internal oxygen levels become insufficient (hypoxia conditions) for all metabolic processes, the cellular energy decreases and accumulation of reactive oxygen species occur (Drew, 1997; Miyashita et al., 2007).

The  $\beta$ -cyanoalanine synthase pathway (CAP) is ubiquitous in all plant tissues (Miller and Conn, 1980). In the typical first step of the pathway, the cyanoalanine synthase (CAS) replaces the sulfhydryl group of a cysteine molecule by cyanide, forming a nitrile (cyanoalanine) and hydrogen sulfide. The following step in the CAP is mediated by a nitrilase enzyme (Nit4), which produces asparagine, aspartic acid, and ammonium (Piotrowski and Volmer, 2006). In the present study, we found that several genes associated with the CAP pathway were up-regulated upon cyanide treatment. Several primary nitrogen metabolism genes were also differentially regulated. A nitrate reductase gene was up-regulated by cyanide application. However previously described experiments demonstrated that nitrate reductase activity is inhibited by cyanide (Shaked et al., 1973). One possible explanation for this observation is that inactivation of the NR proteins by cyanide prompted an up-regulation of the NR gene to compensate. Nitrite reductase gene expression was not affected by cyanide application. Interestingly, the strongest block of nitrogen metabolism appears to be through transport. Several nitrate and ammonium transporters were down-regulated during cyanide application. Though not all nitrate and ammonium transporter genes were affected (and one was even up-regulated) by cyanide application, these results suggest that exogenous application of cyanide can potentially disrupt primary nitrogen uptake. Given the favorable energetics of cyanide conversion to amino acids, the reduced energy cost of limiting ammonium and nitrate uptake, along with the potential down-side of toxicity of cyanide at high levels, it makes sense that plants deal with cyanide through the “path of least resistance” a.k.a the CAP.

To validate and confirm the microarray data, three biological replicates independent from those used for the microarray analysis were used. We selected 15 genes that were significantly up-regulated in the microarray experiments and eight genes that are involved in the cyanide detoxification CAP pathway. The quantitative PCR analysis confirmed the microarray data, and differences between the results obtained from both methods could be attributed to the sensitivity of qPCR or to the differences seen between the dye swap experiments.

In an attempt to find those genes that promptly respond to the cyanide exposure, we carried out a time-course experiment in which *Arabidopsis* plants were exposed to cyanide for various time periods. The results indicated that there were no general expression patterns in response to cyanide treatment at the different time points examined. Several genes responded to cyanide treatment after a 15 to 30 min exposure. As expected, these genes were related to the stress response category such as At3g25760 and At1g07180. The At3g25760 gene encodes for allen oxide cyclase, which catalyzes an essential step in jasmonic acid biosynthesis. Microarray analysis revealed that this gene was up-regulated during arsenic (toxic metalloid) treatment and oxidative stress (Abercrombie et al., 2008). The At1g07180 gene encodes for an internal NAD(P)H dehydrogenase in mitochondria, which acts as an alternative oxidase and its absence causes severe sensitivity to light and drought stress (Ho et al., 2007).

To gain insight into whether bacterial cyanogenesis could have similar effects on *Arabidopsis* gene expression, we studied the expression of 23 genes including those that are involved in cyanide detoxification. The effects of both direct and indirect exposure of *Arabidopsis* plants to strains 15G2 (HCN-producer) and 15G2-*ΔhcnABC* (non-HCN-

producer) were determined. Gene expression data obtained from *Arabidopsis* plants inoculated or exposed to WT strain 15G2 were normalized to those obtained in 15G2-*ΔhcnABC* experiments to eliminate the bacterial effect and only assess the cyanide effects on gene expression. The results obtained from these *in vitro* experiments were different from those obtained by microarray and qPCR when the HCN gas was used. A possible explanation for the low number of responding genes could be due to the slower growth of strain 15G2 on the *Arabidopsis* growing media and incubation conditions (22°C), which could affect the cyanide production by this strain. In addition, our results showed that strain 15G2 produces a low HCN concentration while growing on minimal medium (data not shown).

To address the question whether bacterial produced cyanide in soil is significant, we examined the effects of bacterial cyanogenesis on *Arabidopsis* plants growing in soil. The results showed that eight genes were highly regulated in plants inoculated with WT strain 15G2 when compared to plants inoculated with 15G2-*ΔhcnABC*. In addition, the transcriptional profiles of genes that are involved in cyanide detoxification pathway was similar to that obtained by microarray and qPCR *in vitro* experiments. These results indicated that bacterial cyanide can be absorbed, detoxified and incorporated in the nitrogen pool. These findings raise some questions about how much cyanide the plant can handle. Does the plant send signals for the bacteria to produce cyanide? To eliminate the possibility that differences in gene expression were not due to variations in the bacterial growth rates, we isolated the bacteria at the end of the experiments and calculated their CFU/g of soil. The results showed no significant differences in the growth rates between the WT and the mutant strains, thus changes in gene expression

were likely due to the production of cyanide and not due to growth rate differences between the bacteria used (data not shown). From our results, the At5g5510 and At5g43450 genes that were up-regulated (140 and 72-fold, respectively) upon soil-bacterial cyanide treatment represent successful biomarker candidates for the cyanide production in soil.

Finally, we evaluated the effects of cyanide treatment on the phenotype of 16 mutants of genes that were highly up-regulated in the microarray experiments. Although most of the mutants tested showed a small overall size phenotype before cyanide treatment, five out of the 16 mutants showed distinctive phenotypes upon cyanide treatment. These findings suggest that the knockout of these five genes caused apparent changes in growth and development. Further studies are needed to elucidate the roles of these genes in the response to cyanide treatment.

In summary, data from previous chapters indicated that cyanide produced by *Pseudomonas* sp. strain 15G2 played a role in nematode biocontrol. Here, this study clearly demonstrates that strain 15G2 in fact produces cyanide at the soil level. Several *Arabidopsis* gene markers have been identified as sensors of the cyanide presence. The up-regulation of genes involved in nitrogen metabolism suggested that cyanide can be absorbed by plants and incorporated in the nitrogen pool. Further studies are needed to confirm the incorporation of bacterial cyanide into the plant nitrogen pool and determine whether these cyanides alter nitrate assimilation and nitrogen metabolism in plants.

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# **CHAPTER V:**

## **Nematode gene expression in response to cyanide treatment**

## Contribution of Authorship

All experiments, figures, figure legends, results, discussion, and materials and methods were conducted or created by Haytham Aly unless otherwise noted. Dr(s) Brad Barbazuk and Ruth Davenport conducted the homology search for hypoxia-induced genes in the root-knot nematode genome.

### 5.1 Introduction

Cyanide is an important natural toxicant for both humans and animals. The occurrence of cyanide is relatively widespread, ranging from the simple bacteria and fungi to the higher plants and some animal species. In bacteria, several families such as *Pseudomonas* and *Chromobacterium* produce HCN (Clawson and Young, 1913), while in fungi, cyanide production has been reported in 30 species belonging to five families; *Tricholomataceae*, *Cortinariceae*, *Polyporaceae*, *Agaricaceae*, and *Rhodophyllaceae* (Vennesland et al., 1981). In animals, the Myriapoda and Insecta classes are known to be cyanogenic (Duffy, 1981). In higher plants, there are about 2000 species that are known to produce cyanogenic glycosides such as *Manihot esculanta* (cassava) (Poulton, 1990).

The lethality of cyanide has been attributed to its ability to interfere and inhibit respiration. It binds to cytochrome c oxidase (CcOX) and inhibits oxygen utilization by the tissues and prevents the ATP production during mitochondrial oxidative phosphorylation (Way, 1984). If the CcOX inhibition is not reversed, cyanide continues to interrupt cascades of chemical-cellular reactions and eventually will cause neurological and myocardial dysfunction leading to death (Way, 1984). The insufficient cellular oxygen during the developmental and physiological states is known as hypoxia (Shen et

al., 2005). In mammals, when the oxygen levels become inadequate for the physiological demands, cells respond to that by initiating adaptive responses that are important for survival of the organism. These responses include anaerobic gene expression to increase the energy production, protect cells from oxygen limitation stress, and regulate their survival (Semenza, 2001, 2001).

Several studies have shown that cyanide causes histotoxic hypoxia by preventing the cells from utilizing oxygen and causing oxidative phosphorylation (Van Liew and Chen, 1972; Ruiz, 1975; Edward M. Mills et al., 1996; Prieto et al., 2005). The occurrence of bacterial cyanogenesis in the cystic fibrosis lung has been documented (Ryall et al., 2008; Sanderson et al., 2008). High levels of cyanide were found in cystic fibrosis patients infected with *P. aeruginosa* and were associated with reduced oxygen tensions and an anaerobic environment (Sanderson et al., 2008). The genetic models, *C. elegans* and *Drosophila melanogaster* have been used extensively to study and understand the molecular responses to hypoxia (Scott et al., 2002; Gorr et al., 2004; Shen et al., 2005; Arquier et al., 2006; Anderson et al., 2009).

In response to hypoxia conditions, cells activate various genes such as those encoding for glycolytic enzymes, vasoactive peptides and growth factors to cope with damaging effects resulting from lack of oxygen (Caro, 2001). One of the key factors that regulates gene expression during hypoxia is a family of transcription factors known as hypoxia-inducible factors (HIF) such as HIF-1 (Semenza, 2004). HIF-1 functions as a heterodimer that consists of  $\alpha$  and  $\beta$  subunits. Under normoxia conditions (normal oxygen levels), the  $\alpha$  subunit gets hydroxylated by EGL-9 dioxygenase enzyme followed by binding to the VHL-1 (von-Hippel-Lindau tumor suppressor) protein, which in turn

leads to HIF-1 degradation (Maxwell et al., 1999). The *C. elegans* whole genome expression profile in response to hypoxia conditions is documented (Shen et al., 2005). A set of 110 genes were found to be differentially regulated by hypoxia. Gallagher and Manoil, (2001) showed that *Pseudomonas aeruginosa* PAO1 kills the *C. elegans* by cyanide poisoning and suggested that the killing could be through hypoxia. A mutation in *egl-9*, a gene required for normal egg laying, provides a protection for *C. elegans* from the killing by cyanide poisoning (Darby et al., 1999; Gallagher and Manoil, 2001).

To this end, we decided to investigate a possible role of bacterial cyanide in inducing hypoxia conditions that may lead to the nematode's death. We sought to select a number of genes that were up-regulated under hypoxic conditions (Shen et al., 2005) and determine their transcriptional expression when exposed to cyanide produced by *Pseudomonas* sp. strain 15G2. These experiments aim to: (i) determine whether bacterial cyanide causes the nematode lethality through hypoxia and (ii) to identify homologous gene(s) in the root-knot nematode genome that could be used as biomarker gene(s) to determine if cyanide also acts to kill nematodes in soil.

## 5.2 Materials and Methods

### 5.2.1 Organisms and culture conditions

*C. elegans* wild-type Bristol strain N2 and its *egl-9* mutant (JT 307) used in this study were obtained from the *C. elegans* Genetic Stock Center. Strains were maintained on NGM medium as described in *Chapter II*. Bacterial strains used in this study were *Pseudomonas* sp. strain 15G2 and *E.coli* DH5 $\alpha$ . Media and growth conditions were similar to that described in *Chapter III*.

### 5.2.2 Indirect exposure of *C. elegans* to bacterial cyanide

Nematode paralytic killing assay was carried out as described in *Chapter III* with a few modifications. One hundred freshly hatched L2's of *C. elegans* were placed in 3.5-cm-diameter plates containing NGM medium. After four days, the plates containing mixed stages of the worms were placed opened with an opened plate containing bacteria growing on LB (one-day old) and sealed in a 10-cm-diameter plate. Nematodes were collected in distilled water and frozen in liquid nitrogen. Experiments were done in triplicate. For RKN-J2's, similar experiments were conducted but using 1  $\mu$ M of exogenous HCN.

### 5.2.3 RNA isolation, cDNA synthesis and qPCR analysis

Worms harvested from the above experiments were suspended in 2 mL Triazol (Invitrogen). The RNA isolation was conducted as described by Reinke et al., 2000. Five hundred nanograms of DNase treated RNA were used to generate cDNAs using the SuperScript III First-Strand Synthesis kit (Invitrogen) according to the manufacture's

protocol. For quantification, transcript levels were assayed using Mx3005P- qPCR System (Stratagene) as described in Chapter *III*. qPCRs were conducted in triplicates using gene specific primers (*C. elegans*; Table 5.1 and RKN; Table 5.2) in a 20  $\mu$ L reaction using the Brilliant™ SYBR® Green master mix (Agilent). The *C. elegans* *inf-1* gene was used as an internal control for all the experiments conducted.

### **5.2.3 Identification of putative hypoxia genes from root-knot nematode**

Protein sequences for hypoxia-induced genes from *C. elegans* were compared to the newly released genome (Abad et al., 2008) of the root-knot nematode, *Meloidogyne incognita*, using TBLASTN (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). Best hits (with  $p < 0.05$ ) were identified and primer sequences generated for use in real time PCR (Table 5.2). The RKN 18S rRNA was used as an internal control for all the experiments conducted.



**Table 5.1** Nucleotide sequences for the primers used for *C. elegans* qPCR.

Gene	Description	Primer sequences
<i>egl-9</i>	Dioxygenase that negatively regulates <i>hif-1</i>	<b>BWN:</b> 5'- AACTTGACCTTCGACTCTTCCCGT-3' <b>BWO:</b> 5'- TGAGCGACATTGAAGGTGGAGAGA-3'
<i>vhl-1</i>	von- Hippel-Lindau tumor suppressor protein	<b>BWR:</b> 5'- GGAGCCAGCTCCTCGAATGAATTT-3' <b>BWS:</b> 5'- AGAACGAACGTCCGGCAATTTCTC-3'
<i>hif-1</i>	Hypoxia-inducible transcription factor 1	<b>BWP:</b> 5'- TTCAGGACAACCGAACCGTCGAAT-3' <b>BWQ:</b> 5'- GTAGTTGGCACTGTGTGGCAATGT-3'
<i>cco-1</i>	Cytochrome-C-oxidase 1	<b>BWT:</b> 5'- AAGATGCTTCTCGCTCGTCTTGCT-3' <b>BWU:</b> 5'- ACATGTCCGGAATCTTGCTCACAC-3'
<i>fmo-12</i>	Flavin-containing monooxygenase family member	<b>CXA:</b> 5'- TGATCCAGTTCTTGGTCTTCGCCT -3' <b>CXB:</b> 5'- TTGGTAGGCTCCGGTTTGGTTGTA -3'
W07A12.6	Predicted acyltransferase containing T on B-box	<b>CXC:</b> 5'- CGCAATCGCCACAATCTACGCAAT -3' <b>CXD:</b> 5'- AAATACCTGGCACTCACCACCCTT -3'
F22B5.4	Unknown function	<b>CXE:</b> 5'- TTCTAGATTCCCTTGTCGCCGTCT -3' <b>CXF:</b> 5'- GTCCCATGGTGCTTTGGAATTGCT -3'
<i>inf-1</i>	Initiation factor family member	<b>BWX:</b> 5'- ATCACCACAGACATCTTGGCTCGT-3' <b>BWY:</b> 5'- TCTCGGTGACGAAGTTGATGGCAA-3'

**Table 5.2** Nucleotide sequences for the primers used for RKN- qPCR.

Gene	Primer sequences
CABB01004441.1	<b>DUA:</b> 5'- GGAAATGACTGCTTATAGCACCTT -3' <b>DUB:</b> 5'- CGTAAATATTCGAGCATACCTTTTT-3'
CABB01005905.1	<b>DUC:</b> 5'- CTGTGGCAATGCCCATTTA-3' <b>DUD:</b> 5'- GCCCCACCAACTTTAGCTTT-3'
CABB01000349.1	<b>DUE:</b> 5'- GCGTTTTTCGATTTGGCTAGA-3' <b>DUF:</b> 5'- AGCAAATTCCTTTTCCTGTAATTC-3'
CABB01006580.1	<b>DUG:</b> 5'- TTACTAATTCGATAAGAAGCAGTTTCC-3' <b>DUH:</b> 5'- CAAACCAAATTTGGCCAGAG-3'
CABB01000598.1	<b>DUI:</b> 5'- AGGCCCATAGGGAGAAAAGA-3' <b>DUJ:</b> 5'- TTGGTTGGCCTTCAGTTTTTC-3'
CABB01001892.1	<b>DUK:</b> 5'- TGTTAAATTTCAAACCGTTCCA-3' <b>DUL:</b> 5'- CTGGTGGTCGAATAGATGCTT-3'
CABB01000051.1	<b>DUM:</b> 5'- CCCTTCTCAATCCAGCCATA-3' <b>DUN:</b> 5'- CAAAGCCGTTCTGCTGCTA-3'
CABB01001281.1	<b>DUO:</b> 5'- CCCAGCCTCCTTTGAACTCT-3' <b>DUP:</b> 5'- ACAATATTTTGGTTCACCTATCCAG-3'
CABB01002090.1	<b>DUQ:</b> 5'- CCTGATGAGGCAGTTGCTTA-3' <b>DUR:</b> 5'- CCGAACCCAGACAAAATAG-3'
CABB01001828.1	<b>DUS:</b> 5'- CCATAAATTCAGCACTTCCAAC-3' <b>DUT:</b> 5'- ACAACACCTGGTCGAATGC-3'
18S rDNA (U81578)	<b>DUU:</b> 5'- ACCGTGGCCAGACAAACTAC-3' <b>DUV:</b> 5'- GATCGCTAGTTGGCATCGTT-3'

### 5.3 Results

***C. elegans* qPCR analysis in response to bacterial cyanide.** Mixed stages of *C. elegans* WT (N2) and JT 307 (*egl-9*) mutant strains were indirectly exposed to two bacterial strains 15G2 (HCN-producer) and *E. coli* DH5 $\alpha$  (non-HCN-producer) for one hour. Nematodes were harvested, RNA extracted, and first strand cDNA generated using oligo (dT) primer. The expression levels of seven genes (Table 5.1) were evaluated. Quantitative real time PCR analysis was performed using gene specific primers (Table 5.1). Data obtained were analyzed using the relative fold change ( $2^{-\Delta\Delta C_t}$ ) method and gene expression of nematodes that were exposed to strain 15G2 were normalized to those exposed to *E. coli*. Our results showed that in the *egl-9* mutant strain, all genes tested were down-regulated (Figure 5.1). However, in the *C. elegans* N2 strain, four out of the seven genes tested (*EGL-9*, *FMO12*, *W07A12.6*, and *F22b4.4*) were up-regulated ( $\geq 2$ -fold) (Figure 5.1).

**RKN qPCR analysis in response to bacterial cyanide.** In an attempt to determine if bacterial cyanide has a similar affect on gene expression in the root-knot nematode (RKN), we compared the encoded protein sequence of all *C. elegans* genes that were differentially regulated by hypoxia (Shen et al., 2005) to those of the root-knot nematode (Abad et al., 2008). Unfortunately, we could not find any homologues for the seven genes tested but were able to identify other homologous genes. We chose 10 genes to be evaluated in response to both exogenous cyanide (1 $\mu$ M HCN) and bacterial cyanide produced by strain 15G2. Similar experiments to that described for the *C. elegans* were performed except that second-stage juveniles of RKN were used and gene expression of nematodes that were exposed to strain 15G2 were normalized to those exposed to 15G2-

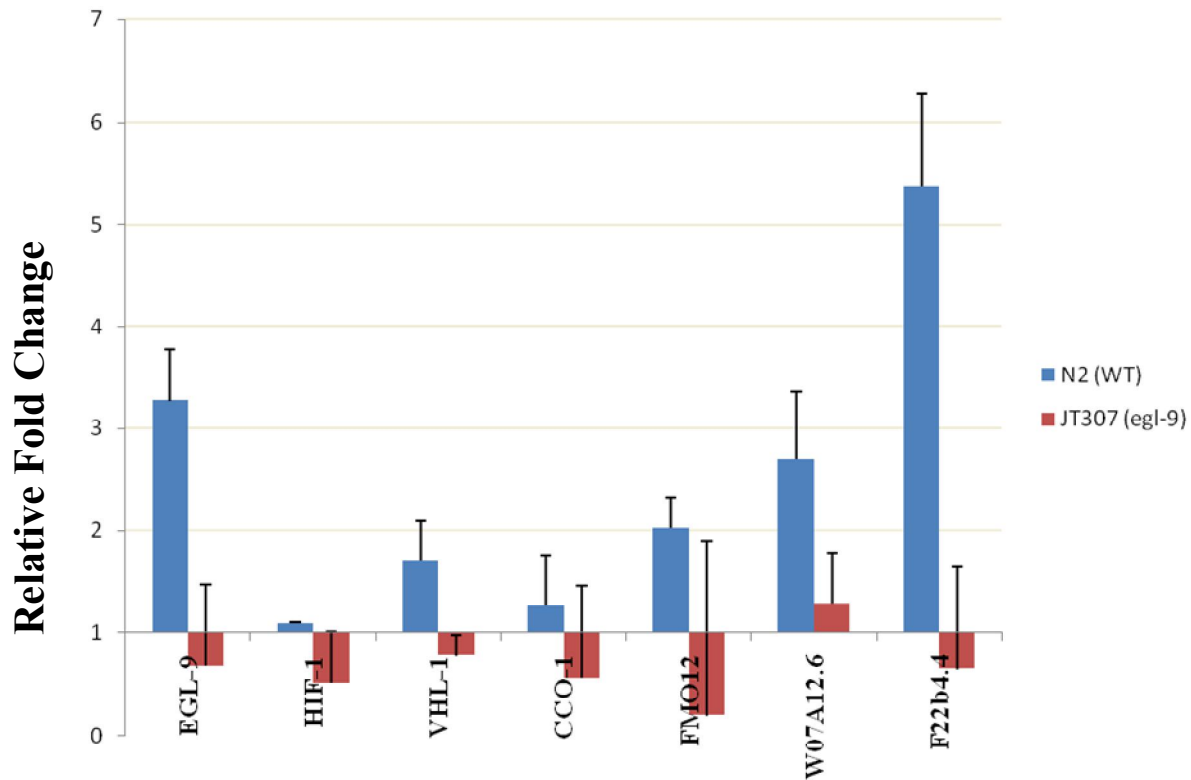
*ΔhcnABC*. Our results showed that no significant differences were observed in the gene expression profile of the genes tested (Figure 5.2). Similarly, exposure of RKN-J2's to exogenous cyanide did not result in major differences in the gene expression profile (Figure 5.2).

## 5.4 Discussion

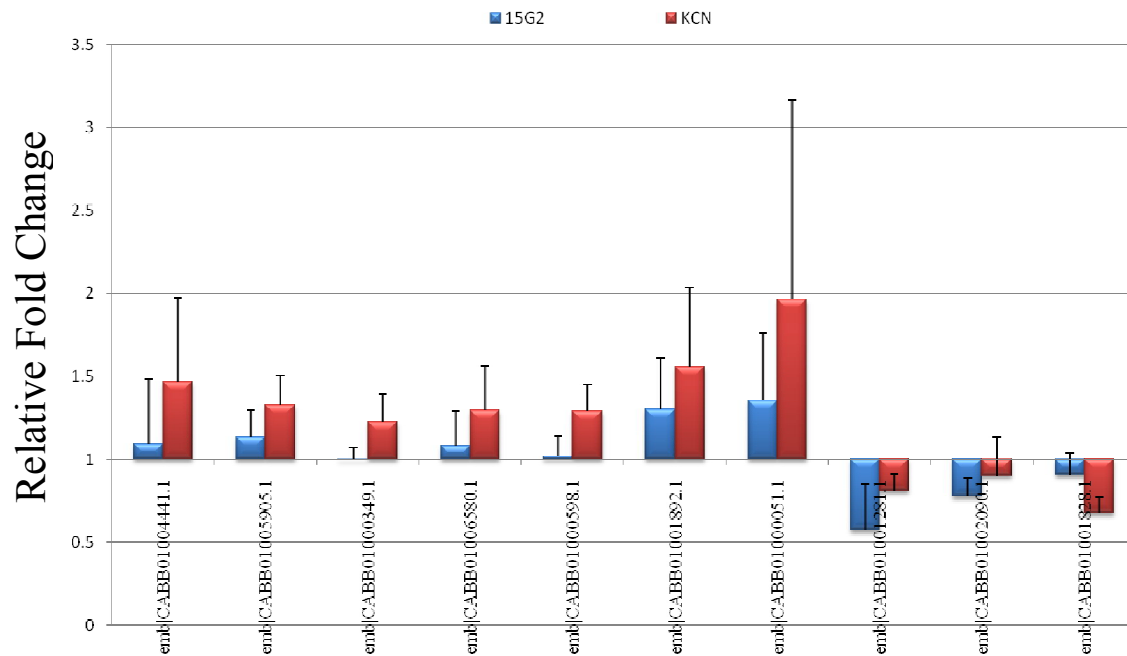
In the present study, we investigated a possible role for bacterial cyanide in inducing hypoxia. Four out of the seven genes tested (*EGL-9*, *FMO12*, *W07A12.6*, and *F22b4.4*) were up-regulated ( $\geq 2$ -fold) upon one-hour exposure to cyanide produced by strain 15G2 (Figure 5.1). These results were in agreement with the hypoxia microarray data (Shen et al., 2005) and differences in the level of gene expression can possibly be explained by the difference in the experimental setup. A proposed model for gene response and hypoxia signaling (Figure 5.3) suggested that under hypoxia conditions, HIF-1 causes and maintains the up-regulation of the *EGL-9* gene, which may cause a negative feedback effect on the HIF-1 activation. In agreement with this model, our results showed that *EGL-9* was up-regulated and HIF-1 was down-regulated under cyanide treatment (Figure 5.1). As expected, the cyanide treatment caused inhibition of cytochrome-c-oxidase (*cco1*) (Figure 5.1). These results further support the speculation that cyanide kills the *C. elegans* through hypoxia (Gallagher and Manoil, 2001).

To further confirm the effect of cyanide on hypoxia induction, we sought to determine whether cyanide has a similar effect on root-knot nematode gene expression. Our data showed no significant differences in gene expression profiles observed upon one-hour exposure to either exogenous cyanide or bacterial cyanide (Figure 5.2). Results obtained from previous experiments (*Chapter III*) showed that indirect exposure of RKN-J2's to strain 15G2 caused the killing of nematodes over a long time period compared to that required for killing the *C. elegans* L2's. The results obtained here may be attributed to the short cyanide exposure time and longer incubation times are required.

In conclusion, the results from the *C. elegans* gene expression experiments demonstrate that cyanide is likely to induce its effect through hypoxia. The effect of cyanide on RKN hypoxia gene expression may require longer exposure time. Alternatively, these results may indicate that cyanide induces the killing through different mechanisms or affects other genes different from those of the *C. elegans*. Both *C. elegans* and RKN microarray studies are required to determine the actual mechanisms through which the cyanide causes its effects and identify nematode biomarkers that could be used to confirm the bacterial cyanogenesis under soil conditions.

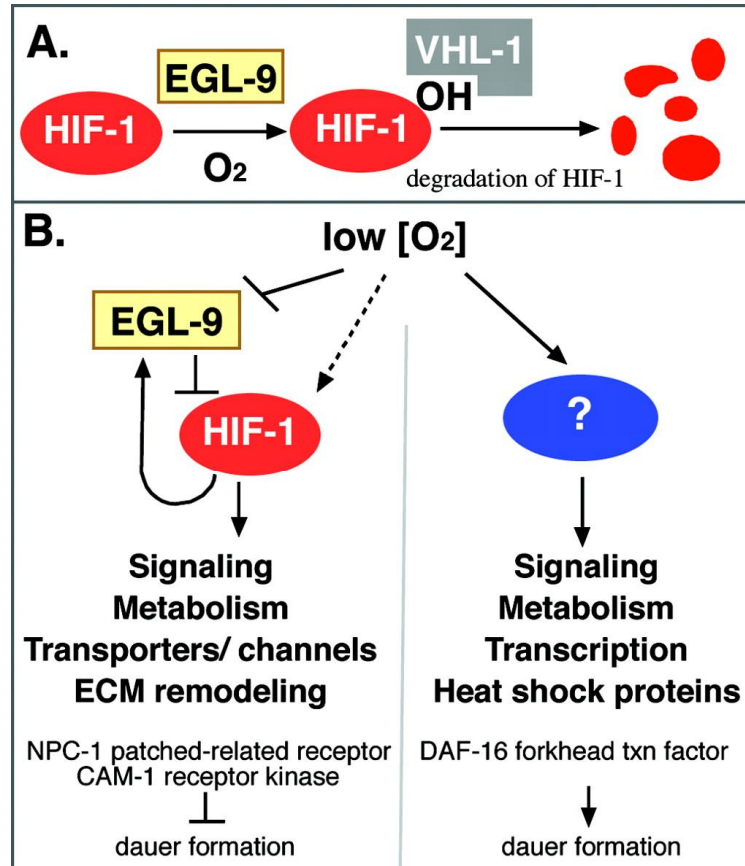


**Figure 5.1** The *C. elegans* quantitative real-time RT-PCR (qPCR) expression analysis for hypoxia-inducible genes. Both the N2 and JT307 *C. elegans* strains were allowed to share the same air with both *Pseudomonas sp.* strain 15G2 and *E. coli* strains for 1 hour. RNA was isolated from the *C. elegans* strains and first strand cDNA was synthesized and subjected to qPCR in triplicates as described in the material and method section. Relative fold change in the expression levels of hypoxia-inducible genes in *C. elegans* exposed to strain 15G2 were normalized to that resulting from exposure to the *E. coli* strain. Error bars representing the standard errors are represented.



**Figure 5.2** The RKN quantitative real-time RT-PCR (qPCR) expression analysis for genes homologous to *C. elegans* hypoxia-inducible genes. Second stage juveniles of RKN were allowed to share the same air with *Pseudomonas sp.* strain 15G2, 15G2-*AhcnABC* strains and with exogenous 1  $\mu$ M HCN for 1 hour. RNA was isolated from the RKN-J2s and first strand cDNA was synthesized and subjected to qPCR in triplicates as described in the material and method section. Relative fold change in the selected genes in RKN-J2s exposed to strain 15G2 or KCN were normalized to that resulting from exposure to the 15G2-*AhcnABC* strain. Error bars representing the standard errors are represented.





**Figure 5.3** Proposed model for genes response to hypoxia. (A) Regulation and degradation of HIF-I under normoxia conditions. (B) Two possible regulatory pathways for HIF-1 in response to oxygen limitation (hypoxia) (Shen et al., 2005).

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# **CHAPTER VI**

## **Summary and Future Directions**

### **Examining the effect of bacterial produced HCN on nematodes**

In an attempt to obtain biological control agents that are capable of antagonizing and suppressing plant-parasitic nematodes, a total of 64 *Pseudomonas* isolates from water and soil samples were screened and determined to be lethal to the free-living nematode, *Caenorhabditis elegans*. We were interested to determine whether all the lethal isolates produce a common molecule(s) through which the bacteria affect the nematode growth and development. Numerous assays that measured motility, exoprotease activity, and production of siderophores, polysaccharides and secondary metabolites (hydrogen cyanide (HCN), 2,4-diacetylphloroglucinol (DAPG), phenazine-1-carboxylate (PCA), pyrrolnitrin and pyoluteorin) were carried out. In addition, the activity of these bacterial isolates against plant-parasitic nematodes (*Meloidogyne incognita* and *Heterodera glycines*), plant-pathogenic bacteria (*Agrobacterium rhizogenes*) and fungi (*Fusarium solani*) were also determined. The results indicated that no single trait accounted for the activity of these isolates against all plant pathogens tested. However, we found that production of HCN was common, being found in 87% of the *C. elegans* lethal isolates and 100% of the plant-parasitic nematode lethal isolates. Based on these results we hypothesized that HCN is one of the main mechanisms used by our *Pseudomonas* isolates to antagonize and/or kill plant-parasitic nematodes.

To test the hypothesis that HCN is the lethal component responsible for reducing the parasitic load of plant-pathogenic nematodes on plants in our experiments, we chose one of our *Pseudomonas* strains for further characterization. *Pseudomonas* strain 15G2 was chosen for further analysis because: 1) This strain possesses activities against several plant pathogens such as *Agrobacterium rhizogenes* and plant-parasitic nematodes,

2) it is a motile bacterium that may actually represent an important fitness trait for studying the activity against migratory stages of nematodes and/or other phytopathogens, and 3) the strain possesses several biochemical characteristics such as production of exoproteases, siderophores, polysaccharides, DAPG and HCN commonly seen in many *Pseudomonas* used as biocontrol agents.

In order to identify the nematode-lethal mechanism(s) in 15G2, nearly 2,700 transposon knock-outs were generated and tested for their ability to kill nematodes. Despite the differences between *C. elegans* and plant-parasitic nematodes, our lethality screening experiments (see *Chapter II*) indicated that *C. elegans* was a useful tool for identifying bacteria with lethality toward plant-parasitic nematodes. Thus, the activities of the transposon knock-out mutants were initially tested against *C. elegans*. We identified 74 mutants that were no longer lethal to *C. elegans* in the *in vitro* assay. DNA sequence analysis of regions flanking the transposon indicated that these mutants were in genes encoding for metabolic (e.g. lactate dehydrogenase, NADP transhydrogenase), regulatory (e.g. LysR transcriptional regulator, histidine kinase sensor protein) and structural (e.g. amino acid transporters, motility protein) proteins. Based on sequence comparison of these genes to existing genomic sequences of other *Pseudomonas*, strain 15G2 was found to be closely related (90-95% similarity) to the biocontrol strain *Pseudomonas fluorescens*, Pf-5. Regardless of the DNA sequence similarities between strain 15G2 and Pf-5, our *C. elegans* assays showed that the Pf-5 strain was not lethal to nematodes.

We tested the ability of the transposon knock-out mutants to produce HCN. Numerous 15G2 non-lethal knock-outs with growth rates comparable to that of WT

showed a 10-fold reduction in HCN production. The complementation of three of these mutants (see *Chapter III*) with the corresponding WT genes restored their ability to produce HCN. These results suggest that the genes identified from the transposon mutagenesis may be involved in directly or indirectly regulating HCN biosynthesis, but how they are involved still remains to be determined.

Interestingly, none of the generated transposon knock-out mutants (with HCN<sup>-</sup> phenotype) were in the well-known *hcnABC* operon. We cloned and sequenced the *hcn* operon from 15G2 using primers made to the highly conserved regions in the HCN operon and collected 5' and 3' flanking regions using PCR genome walking techniques. Using Southern blots we determined that only one *hcnABC* operon exists in 15G2. Using the homologous recombination constructs  $\Delta hcnA$ ,  $\Delta hcnB$ ,  $\Delta hcnC$ , and  $\Delta hcnABC$ , deletion mutants in the *hcnA*, *hcnB*, *hcnC*, and *hcnABC* genes, respectively, were created. The growth rates of these mutants were not significantly different from that of the wild-type strain. These mutants produced a very low level of HCN (<5  $\mu$ M), which according to other studies, are considered non-HCN producers (Laville et al., 1998). The disruption of any of the *hcnABC* genes leads to a 90% reduction in HCN production, suggesting that these genes may form a biosynthetic pathway. The low levels of cyanide (<5  $\mu$ M) production in the deletion mutants may also suggest the presence of another mechanism(s) through which the remaining 10% of HCN production occurs. We also found that the expression of either *hcnB* or *hcnC* using a constitutive *Bacillus* promoter induced lethality in *E. coli*, indicating that *hcnB* or *hcnC* by itself can produce HCN or other lethal byproducts. It is not known whether *hcnB* and *hcnC* together are sufficient for HCN production in *Pseudomonas* sp. strain 15G2.



Additionally, the effects of the 15G2 hcn deletion and transposon mutants on *C. elegans* were examined. As expected, indirect exposure of *C. elegans* to these mutants did not result in killing the nematode. However, when 15G2 HCN mutants were in direct contact with *C. elegans*, they caused lethality over a longer time period (5 days) as compared to wild-type 15G2 (1 day). These results strongly suggest that HCN is one of the major contributing factors to *C. elegans* lethality in our assays. When HCN is present, the effect on killing the nematode occurs in hours. However, if HCN is low or absent, HCN or other substances may build up over time in the medium.

To further evaluate the role of HCN in biological control of plant-parasitic nematodes in the real world (soil), we studied the effects of the wild-type strain 15G2, and its deletion mutants,  $\Delta hcnA$ ,  $\Delta hcnB$ ,  $\Delta hcnC$ , and  $\Delta hcnABC$  on the soybean cyst nematode. In addition, we tested two of the mutants contain transposon insertions in their genes and were impaired in HCN production. Using the wild-type 15G2 strain, we observed a marked reduction in the nematode population, whereas all mutants exhibited a reduced capacity for protecting the plants from the soybean cyst nematode. These results indicate that HCN is likely the dominant factor in 15G2's biocontrol capacity toward the soybean cyst nematode. Other studies have shown that HCN produced by *P. fluorescens* CHA0 contributes to the bacterium biocontrol activity against the fungal black root-rot disease (Voisard et al., 1989; Laville et al., 1998), *Meloidogyne* spp. (Siddiqui and Shaukat, 2002; Siddiqui et al., 2006).

## **Examining HCN induced gene changes on plants and nematodes**

To further support our hypothesis that bacterial produced HCN provides protection from plant-parasitic nematodes in the soil we wanted to provide evidence that bacteria are capable of producing HCN in the soil. To this end we decided to use a gene marker system to address whether organisms are sensitive to HCN levels in the soil. *Pseudomonas* spp. are well known for their ability to colonize plant roots and provide protection from other plant pathogens including nematodes. We chose to examine HCN regulated gene expression in plants inoculated with *Pseudomonas* as well as within the nematode itself. These studies provided additional evidence that HCN is being produced at the soil level and accounts for the nematode lethality.

### ***HCN regulated gene expression in Arabidopsis***

We conducted a microarray study to examine gene expression in response to HCN treatment in *Arabidopsis*. To our knowledge, this microarray study is the first microarray study to evaluate the effect of cyanide treatment on *Arabidopsis* genome expression. One month old *Arabidopsis* roots were exposed to a sublethal dose of HCN for two hours and the transcriptional profile determined using the Agilent ATH1 Microarray chips. ANOVA analysis (p-value <0.05) of the microarray results identified approximately 2300 genes that were differentially regulated during the two hour exposure to HCN. We identified about 1000 genes that were up-regulated (> 2-fold) and 1200 genes that were down-regulated (<-2). The analysis of the differentially regulated genes using The Gene Ontology (GO) database revealed that the largest classes of HCN-regulated genes belong to those involved in response to reactive oxygen, hypoxia, stress or genes with 'unknown

function'. The analysis of genes associated with nitrogen metabolism and CAP pathways (cyanide detoxification pathway) also showed several genes that were differentially regulated during HCN exposure. These results suggested that a portion of the cyanide absorbed by the roots is potentially detoxified and incorporated into the plant's endogenous nitrogen pool. Twenty three genes were used in real time RT-PCR to validate the microarray data obtained.

A time-course experiment was conducted to determine if there is any correlation between transcriptional responses and cyanide treatment. Results showed that numerous genes responded differently to HCN but no common pattern or induction of expression was observed. For example, changes in transcriptional mRNA levels of several genes were detectable after 15 min exposure to HCN while changes in others were only detectable after 2 hours of cyanide exposure (see *Chapter IV*). Based on the microarray, time point experiments, and real time RT-PCR analysis, two conclusions can be drawn. First, some genes respond promptly to the cyanide exposure and might be useful as biomarkers for HCN production by bacteria in soil. Second, the differential regulation of genes associated with both nitrogen metabolism and cyanide detoxification pathways (CAP) suggest that plants may convert cyanide to usable nitrogen forms such as amino acids.

To further validate our results, similar experiments were conducted using the 15G2 strain and its *hcn* deletion mutants as the source of cyanide. *Arabidopsis* plants growing in soil were inoculated with different wild-type and mutant 15G2 strains for one week. Roots were harvested, RNA isolated and gene expression examined using real time RT-PCR. Our results showed that eight out of the 15 HCN regulated-genes tested

were up-regulated in plants inoculated with the wild-type strain of 15G2 and that high levels of expression were dependent on having an intact *hcn* operon. Additionally, genes associated with the nitrogen metabolism and CAP pathways were also regulated and dependent on whether an intact *hcn* operon was present. Taken together, we conclude that HCN produced by strain 15G2 largely contributes to the nematode biocontrol in the soil. Additionally, we were able to identify several *Arabidopsis* genes that will be potentially useful as biomarkers for HCN under soil conditions.

### ***HCN regulated gene expression in nematodes***

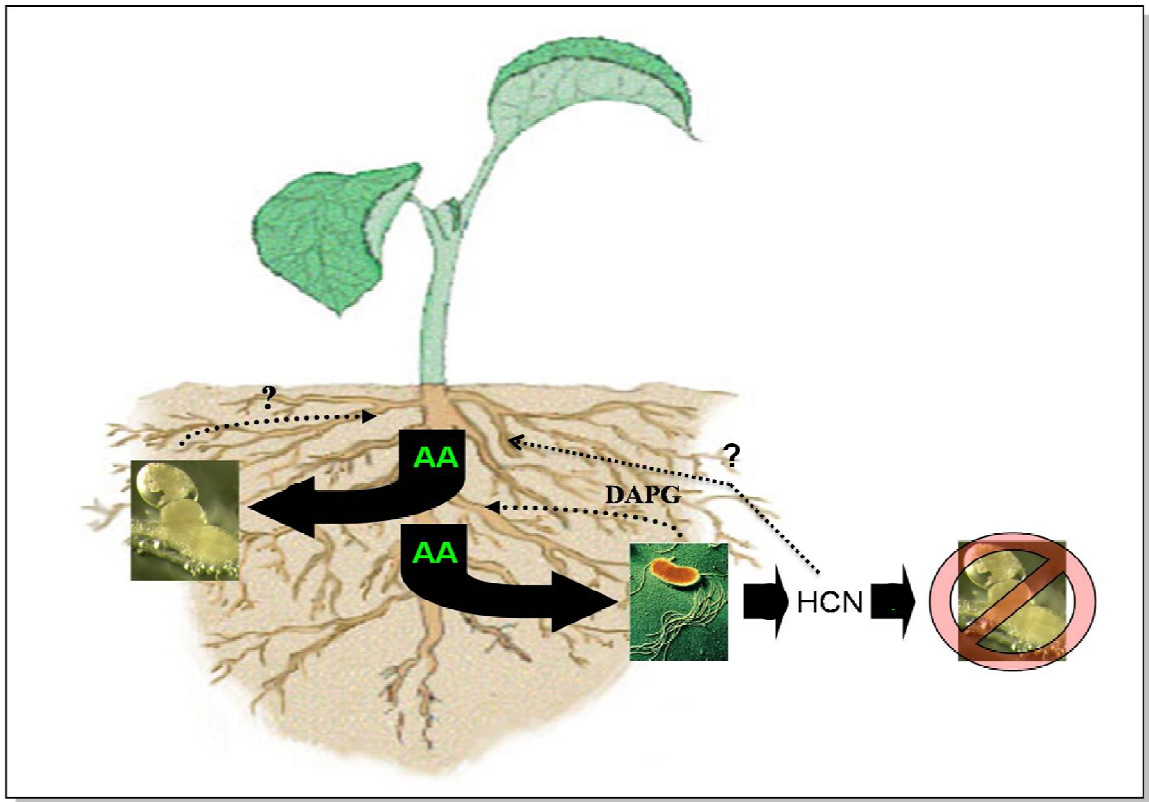
A second way to measure HCN production in the soil was to examine HCN-induced gene expression in the target organism, namely the nematode. A previous study has shown that exogenous exposure to HCN was lethal to *C. elegans* and suggested that the killing may be through hypoxia (Gallagher and Manoil, 2001). The transcriptome profile of the *C. elegans* in response to hypoxia is well documented (Shen et al., 2005). We chose seven genes from the microarray analysis performed by Shen et al., (2005) that were differentially regulated during hypoxia for evaluation under our experimental conditions. Our quantitative real time PCR analysis indicated that the indirect exposure of *C. elegans* to strain 15G2 produced a similar expression pattern to that obtained by the microarray study (Shen et al., 2005). Comparison of hypoxia-induced genes in *C. elegans* to the recently release genome of the root-knot nematode, *Meloidogyne incognita* identified several putative hypoxia-regulated genes for the root-knot nematode. Examination of these putative hypoxia-regulated genes in the root-knot nematode juveniles challenged with WT strain 15G2 failed to show any HCN regulation. These results suggest that the bacterial cyanide may induce hypoxic conditions that could lead

to *C. elegans* lethality. However these results did not provide any evidence that the killing is exclusively through hypoxia, and thus cyanide may have alternative effect(s) through other mechanisms. Furthermore, the putative hypoxia-regulated genes from the root-knot nematode may not respond to HCN or hypoxia like that of *C. elegans*. More work is needed to identify better marker genes in the root-knot nematode genome and further studies are needed to examine whether HCN is solely a hypoxic inducing condition or whether HCN has other specific effects that lead to lethality.

#### ***A new model for HCN in the rhizosphere***

Based on all findings obtained from these studies, we propose a new hypothesis and model (Figure 6.1) for the role of bacterial produced HCN. The hypothesis is that HCN produced by *Pseudomonas* sp. strain 15G2 provides protection to plants from plant pathogens in the soil but also enhances plant growth by providing a nitrogen source. In this model, plants provide organic nitrogen (such as amino acids and proteins) through the root exudates, which are broken down and absorbed by the bacteria. Upon absorption, bacteria use the amino acids as precursors for HCN production. HCN is released into the rhizosphere along with the biocontrol compound 2,4-diacetylphloroglucinol (DAPG) synthesis. DAPG inhibits the influx of amino acids across root cells (thereby increasing amino acid concentrations in the rhizosphere) allowing for more HCN to be produced. A portion of the cyanide produced by the bacteria is reabsorbed by the roots, detoxified and converted via the CAP pathway to plant-usable forms of nitrogen such as the amino acid asparagine.

Several lines of evidence support this hypothesized model. Naturally, plant roots cycle amino acids through their plasma membrane and the net efflux is known as root exudates. The normal concentrations of amino acids in the root exudates are between 0.1 to 10  $\mu\text{M}$  (Jones and Darrah, 1994). Treatment of alfalfa roots with 200  $\mu\text{M}$  of DAPG inhibited the amino acid influx and increased the concentrations of 16 amino acids in the root exudates (Phillips et al., 2004). Amino acids alanine, proline, arginine, asparagine, glycine, and glutamate were present at highest concentrations (Phillips et al., 2004). Our results showed that strain 15G2 is capable of producing HCN at the soil level (see *Chapter IV*) and can produce HCN from different amino acid precursors such as: proline, alanine, arginine, asparagines, aspartate, glutamine, glutamate, histidine, and serine (see *Chapter III*). In addition, the bacterium was able to use very low concentrations (1  $\mu\text{M}$ ) of amino acids for their growth and hydrogen cyanide production (data not shown). Furthermore, strain 15G2 has the *phlD* gene encoding for DAPG (see *Chapter II*) and likely produces and secretes DAPG into the rhizosphere. The results from this study and others have shown that HCN plays a role in the direct biocontrol of plant pathogens (Laville et al., 1992; Siddiqui and Mahmood, 1996; Siddiqui et al., 2006). On the other hand, our microarray and qPCR analysis for *Arabidopsis* gene expression in response to either exogenous or bacterial HCN, showed that genes associated with nitrogen metabolism and cyanide detoxification pathway are also up-regulated (see *Chapter IV*). Further studies are needed to examine this hypothesis, and determine: (i) the level of bacterial cyanide that plants can handle, (ii) the level of bacterial cyanide incorporation into plant nitrogen pools, and (iii) evaluation of DAPG mutants and their ability to alter HCN production in the soil.



**Figure 6.1** Model for the strain 15G2-plant interactions.

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