Chapter 1

General Introduction
Rhizobacteria are an important functional group of soil ecosystems. Plant rhizosphere has an environment that is substantially different from the adjacent soil. The rhizosphere typically has intense microbial activity and high populations of bacteria compared with non-rhizospheric soil. Plant root exudates may contain up to 20% of root allocated carbon and metabolically active cells. This leads to a highly evolved relationship between the plant and rhizosphere microorganisms (Handlesman and Stabb, 1996). Because plant exudates play a crucial role in the chemical composition of the rhizosphere, significant differences may be found between rhizosphere of individual plants (Yang and Crowley, 2000). Consequently, the microbial community of each rhizosphere would be different from the microbial community of an adjacent rhizosphere. Indeed the microbial community in the rhizosphere of a given plant can be and is often very different from the microbial community in the rhizosphere of another plant (De Leij et al., 1994; Griffiths et al., 1999). Several studies have shown that plants may influence the microbial species composition in their rhizosphere through their root exudates (Lupwayi et al., 1998; Mahaffee et al., 1997; Smalla et al., 2001; Kowalchuk et al., 2002; Costa et al., 2006).

Because plants can influence soil microbial species composition, plants actively select the microbial species that can grow in their rhizosphere (Doornbos et al., 2012). The selected microorganisms are often beneficial groups such as mycorrhizas, nitrogen fixers, phosphate solubilizers, plant growth promoters and microorganisms that can protect plant against pests (Abou-Aly et al., 2006; Picard and Bosco, 2008). Most rhizospheric microorganisms may have dual roles such as nutrient uptake and protection against pests and pathogens. For instance, mycorrhizas promote plants growth through uptake of nutrients and also provide protection against a range of root pathogens (Dodd, 2000). An important group of rhizospheric bacteria are the Plant growth promoting rhizobacteria (PGPR). PGPR promote plant growth through facilitation of nutrient uptake and by production of phytohormones and ACC-deaminase synthesis. PGPR can also indirectly promote plant growth through the suppression of pathogen growth by various means such as release of siderophores, antibiotics, HCN and cell wall degrading enzymes like chitinase. Species of *Azotobacter*, *Bacillus*, *Burkholderia*, *Azospirillum*, *Alcaligenes*, *Serratia*, *Erwinia* and *Pseudomonas* are some of the common PGPRs (Soltani et al., 2010).
The pseudomonad bacteria are an important group of soil bacteria and play a prominent role in the interface between soil and plant roots (Ramette et al., 2003). Pseudomonad species like *Pseudomonas aeruginosa* and *P. putida* are soil bacteria that can be pathogenic to humans and animals (Graevenitz and Weinstein, 1971; Darby et al., 1999). For instance, *P. aeruginosa* can cause cystic fibrosis in humans (Govan and Deretic, 1996; Hare et al., 2012). Other pseudomonad species like *P. fluorescens* are important plant growth promoting bacteria. *P. fluorescens* is known to produce plant growth hormones like siderophores and indole acetic acid (IAA) (Dey et al., 2004). Alleviation of disease or pest attack also indirectly promotes plant growth. Rhizospheric pseudomonads exhibit antagonistic effects toward fungal pathogens of plant roots (Ramette et al., 2003). Pseudomonad bacterial species are among the most widely studied group of antibiotic producers in the rhizosphere (Handelsman and Stabb, 1996). *P. fluorescens* strains produce secondary metabolites like pyoluteorin; pyrrolnitrin; 2, 4-diacetylphloroglucinol and hydrogen cyanide, which can inhibit the growth of several pathogens and pests and contribute to disease suppression and healthy plant growth (Handelsman and Stabb, 1996; Haas and Défago 2005). For instance, *P. fluorescens* CHA0 has been shown to suppress diseases caused by soilborne phytopathogenic fungi such as *Fusarium oxysporum*, *Gaeumannomyces graminis* var. *tritici*, *Pythium ultimum*, *Rhizoctonia solani*, *Aphanomyces euteiches* and *Thielaviopsis basicola* (Voisard et al., 1989; Troxler et al., 1997; Siddiqui and Shaukat, 2002; Ramette et al., 2003; Ramette et al., 2006; Sari et al., 2008). The main mechanism of bacterial action is believed to be by the direct inhibition of fungi by bacterial HCN (Blumer and Haas, 2000). This is comparable to HCN-mediated plant defence mechanisms against herbivores (Luckner, 1990; Ramette et al., 2003). Indeed, HCN producing *P. fluorescens* CHA0 has been shown to kill even insect pests like termites under *in-vitro* conditions (Devi and Kothamasi, 2009). Production of HCN by pseudomonad bacteria is an important mechanism of disease suppression in the plant host and is recognized to be of significance for effective biocontrol (Ramette et al., 2003). However, a deleterious effect of microbial HCN has been reported in several plant species (Bakker and Schippers 1987; Alström and Burns 1989; Kremer and Souissi, 2001; Owen and Zdor, 2001; Kremer, 2006; Flores-Vargas and O'Hara, 2006). The difference between biocontrol and deleterious strains is a consequence of differences in patterns of HCN production on plant surfaces, inside plant tissues, or both (Ramette et al., 2003).
HCN production has been reported among plants where it is believed to constitute a chemical defence against herbivores and pathogens (Blumer and Haas, 2000; Francisco and Pinotti, 2000; Zagrobelny et al., 2004; Ballhorn et al., 2009). HCN production also occurs in fungi, algae and bacteria. Microbial cyanide production may have an ecological role. HCN accounts for a part of the biocontrol capacity of cyanogenic bacteria such as \textit{P. fluorescens} CHA0. Bacterial cyanogenesis is not very widespread and may be restricted to proteobacteria \textit{Chromobacterium violaceum} and fluorescent pseudomonads. Recent studies have shown that other bacterial species like \textit{Rhizobium leguminosarum}, \textit{R. radiobacter}, \textit{Alcaligenes latus}, \textit{Aeromonas caviae} and \textit{Burkholderia cepacia} can also produce HCN (Antoun et al., 1998; Devi et al., 2007; Ryall et al., 2008). Bacterial HCN is formed stoichiometrically from glycine through an oxidative reaction (Figure 1.1) catalysed by a membrane bound HCN synthase enzyme complex (Laville et al., 1998; Blumer and Haas, 2000).

![Figure 1.1. HCN synthesis from glycine is catalysed by HCN synthase enzyme complex (Blumer and Haas, 2000; Laville et al., 1998).](image)

The systemic name cyanide-forming glycine dehydrogenase (EC 1.4.99) has been proposed for HCN synthase based on sequence similarities with known dehydrogenases and oxidases (Blumer and Haas, 2000). HCN production is highest during the transition from the exponential to the stationary phase (Blumer and Haas, 2000; Devi et al., 2007). This idiophase is followed by a rapid loss of HCN synthase activity (Blumer and Haas, 2000). HCN production is strongly regulated by the ANR protein (anaerobic regulator of arginine deaminase and nitrate reductase). ANR mutant strains of \textit{P. aeruginosa} and \textit{P. fluorescens} produce very low amounts of HCN (Laville et al., 1998). ANR belongs to the FNR (fumarate and nitrate reductase regulator) family of transcriptional regulators (Blumer and Haas, 2000). The promoter
of hcnABC genes of *P. fluorescens* CHA0 contains an FNR/ANR box in the −40 region, which is characteristic of promoters activated by FNR or ANR (Blumer and Haas, 2000). Induction of *hc* expression by oxygen limiting conditions is influenced by ANR and the ANR box. HCN synthase is regulated by oxygen tension at two levels: on the ANR during transcription and at the level of enzyme activity.

Global regulatory elements coordinate production of secondary metabolites in pseudomonads (Handelsman and Stabb, 1996). The global activator GacA, a response regulator, positively regulates the synthesis of HCN and other secondary metabolites (Laville *et al*., 1998). Indeed, GacA-negative mutants of *P. fluorescens* CHA0 are pleiotropically defective in HCN synthesis (Laville *et al*., 1998). Glycine is the immediate precursor of cyanide in proteobacteria. Cyanide is derived from the methylene carbon of glycine and CO₂ from the carboxyl group of glycine in *C. violaceum*, *P. fluorescens* and *P. aeruginosa* (Askeland and Morrison, 1983; Blumer and Haas, 2000). Three structural genes hcnA, hcnB and hcnC that encode for HCN synthase have been reported from *P. fluorescens* CHA0 and *P. aeruginosa* PAO1 (Laville *et al*., 1998; Pessi and Haas, 2000). The three genes are sufficient to render *Escherichia coli* cyanogenic when expressed from a T7 promoter (Laville *et al*., 1998). A relationship exists between the ability of biocontrol pseudomonads to produce HCN and their biocontrol capacity (Ramette *et al*., 2003). Polymorphism in the nucleotide sequence of the three genes may affect HCN production and consequently the biocontrol ability of the bacterial strains. Recent studies have shown that polymorphism in the HCN synthase gene actually does influence HCN production (Ramette *et al*., 2003; Devi *et al*., 2012). While Ramette *et al*., (2003) studied the polymorphism in partial hcnBC gene sequences derived from HCN producing pseudomonads of worldwide origin, Devi *et al*., (2012) analysed polymorphism in partial hcnAB gene sequences of pseudomonads from Delhi. The latter study focussed on a narrower geographic scale. In spite of the differences in scale of study, both investigations showed that polymorphism in the HCN synthase gene is high and divergence in the gene sequence can lead to ecologically distinct species (Ramette *et al*., 2003; Devi *et al*., 2012). The formation of ecotypes among HCN producing bacterial populations appears to be dependent on local abiotic factors.
Cyanide producing pseudomonad bacterial species

(Devi et al., unpublished data). Sequence information of the HCN synthase gene consequently can serve as a useful tool for the selection of suitable strains for biocontrol applications.

HCN producing bacteria as mentioned previously have found application as biocontrol agents. Most studies involving HCN producing bacteria have been against fungal pathogens (Voisard et al., 1989; Troxler et al., 1997; Siddiqui and Shaukat, 2002; Ramette et al., 2003; Ramette et al., 2006; Sari et al., 2008). A few studies have also investigated biocontrol ability of HCN producing bacteria on other pathogenic organisms. Caenorhabditis elegans – a root pathogenic nematode was killed by HCN producing P. aeruginosa (Gallagher and Manoil, 2001) Meloidogyne javanica was killed by P. fluorescens CHA0 through cyanide poisoning (Siddiqui et al., 2006). HCN producing bacteria have been found to be effective in killing subterranean termite Odontotermes obesus under in-vitro conditions (Devi et al., 2007). Although use of biocontrol agents against termite pests can be very appealing from an environmental point of view, biocontrol agents such as pathogens, parasites and predators have not been effective against termites. Use of chemical pesticides remains the chief means of control of termite infestation. This is because of the behavioural adaptations of social insects like termites. Pathogens and parasites while being effective biocontrol agents against other insect pests are not successful against termites because pathogens are removed by grooming and infested members are isolated from the colony. HCN producing bacteria can offer an option to overcome the behavioural adaptation of termites because no individual from the termite colony is actually infected. The strategy proposed is similar to the one used by pyrethrin based insecticides. Pyrethrin is a neurotoxin and kills all insects on contact (Yang et al., 2012). HCN is a respiratory toxin that inhibits cytochrome oxidase in the respiratory electron transport chain and causes death. The strategy is to introduce inoculum of HCN producing bacteria into termite colonies and cause death of termites not through infection but by the release of HCN. Nevertheless, HCN is a potent toxin and there would be concerns about the possible effects on mammals and other higher animal groups. HCN producing bacteria are fairly prevalent in the soil, yet there have been no reports describing acute or chronic toxicity caused by bacterial cyanide in animals or
plants. This is because bacterial cyanide production is very tightly regulated such that local cyanide concentrations are usually below 1 mM and can be tolerated by many living cells (Blumer and Haas, 2000). However, a massive increase in the number of cells producing HCN can lead to deleterious effects. This is the strategy aimed at when dealing with termites. Termite mounds offer a structural advantage for use of HCN producing bacteria as biocontrol agents. If inoculum is introduced into the confined spaces of a termite mound/infestation, the HCN production will be restricted to the spaces within the nest and kill the termites (Figure 1.2) but at the same time will not present any danger to other groups of organisms.

Figure 1.2. Termite mound of *O. obesus* at Bhatti mines, Delhi. Introduction of HCN producing bacterial inoculum into the confined spaces of the termite mound could offer a viable biocontrol option.
Indeed, a similar advantage is presented by plant rhizospheres. HCN producing bacteria like *P. fluorescens* and *P. putida* are aggressive root colonizers (Troxler *et al.*, 1997). The HCN released by them in the rhizosphere of their hosts is in sufficient concentrations to kill pathogenic fungi such as *Thielaviopsis basicola* in soil microcosm experiments (Troxler *et al.*, 1997). In my studies with termite *O. obesus*, I found that HCN production accounted for a substantial part of biocontrol ability of the pseudomonad strains isolated and analysed in this study. Cyanide inhibits cytochrome c oxidase (CCO), the terminal component of the respiratory chain in many organisms (Blumer and Haas, 2000). When termites were incubated with HCN producing bacterial strains, CCO of the termite respiratory chain was inhibited (Devi and Kothamasi, 2009). Termites incubated with a HCN– mutant *P. fluorescens* CHA77 strain (which did not produce HCN) did not suffer CCO inhibition (Devi and Kothamasi, 2009). My pilot study to test the efficacy of HCN producing bacteria as effective biocontrol agents against termite pests employed HCN producing bacteria of different genera (Devi *et al.*, 2007; see chapter 2). However, I subsequently narrowed my study to pseudomonad species in order to understand the functioning of the HCN synthase gene and to analyse how gene divergence can lead to development of ecologically distinct populations at each niche. This thesis was framed with the following objectives:

1. To study whether HCN producing bacteria can effectively kill insect pests such as termite *O. obesus*.

2. Analysis the of effect of bacterial HCN on termite cytochrome c oxidase.

3. Study of the diversity in HCN producing pseudomonad bacteria on local geographic scale from the Delhi region.

4. Isolation of a partial *hcnAB* gene and analysis of whether sequence divergence in the gene could lead to the establishment of ecologically distinct populations.
References


