Chapter 1
Literature review
and Introduction

1 Introduction

Pathogenic microorganisms which affect the plant health are a major and chronic threat to food production and ecosystem stability worldwide. In most agricultural ecosystems, soil-borne plant pathogens are a major limitation in crop yields. They are also difficult to control compared to the pathogens that attack the above-ground parts of the plant. Soil-borne pathogens are adapted to grow and survive in the bulk soil, but the pathogen encounters the plant and establishes a parasitic relationship in the rhizosphere (Raaijmakers et al. 2009). Plant pathogens can be broadly divided into two types: Necrotrophs- those that kill the host and feed on the contents and Biotrophs- those that require a living host to complete their life cycle (Dangl and Jones 2001). Mostly Gram negative bacteria in the *Pseudomonadaceae* and *Enterobacteriaceae*, are necrogenic which are able to cause the death of plant cells. They secrete cell wall degrading enzymes or produce virulence proteins that eventually kill the plant cells. These pathogens colonize the apoplast and produce the rots, spots, wilts, cankers, and blights affecting virtually all crop plants (Alfano and Collmer 1996). The development of substantial pathogen population is often an important factor in the diseases caused by necrogenic Gram negative bacteria since they use “quorum sensing”. Quorum sensing is a cell to cell signalling mechanism that regulates virulence gene expression and is dependent on population density (Fuqua et al. 1994).

1.1 Quorum sensing: an overview

Quorum sensing is a cell-cell communication mechanism by which bacteria count their own numbers by responding to the accumulation of a signalling molecule that they have produced and secreted into their environment (Bassler and Losick 2006). Quorum sensing controlled behaviors only occur when bacteria are at high population densities and become effective by the simultaneous action of a group of bacterial cells. When undertaken by an individual bacterium, these behaviors are not useful and are costly. Depending on the bacterial strain various bacterial processes can come under the control of the quorum sensing. Processes such as bioluminescence, the secretion of virulence factors, the production of public goods and the formation of biofilms are some of the examples (Bassler 2002). Most Gram negative bacteria use LuxI/LuxR type of quorum sensing systems. LuxI/LuxR quorum sensing system regulating bioluminescence was first identified in the marine bacterium *Vibrio fisheri* (Hastings and Greenberg 1999).

Quorum sensing involves the production and release of small, diffusible, low molecular weight, extracellular signalling molecules known as autoinducers. In Gram negative bacteria, the autoinducer is an acylated homoserine lactone (AHL). AHL is synthesized cytoplasmically and diffuses down its concentration gradient through the bacterial membrane and accumulates both intra- and extracellularly in proportion to cell density. As the bacterial population density increases the local concentration of AHL also increases. Once a threshold concentration of AHL, or population size, is achieved the cognate quorum sensing receptor is bound effectively by AHL and undergoes activation. This results in the expression of quorum sensing regulated genes (Fuqua et al. 2001; Miller and Bassler 2001). Basal level AHL synthesis is maintained by AHL synthases. S-adenosylmethionine and acyl-acyl carrier protein, a fatty acid biosynthesis intermediate, are the substrates for AHL synthase. The enzyme belongs to LuxI AHL synthase family while the specific AHL receptors are LuxR family transcriptional regulators. LuxR family members consist of two domains, a C-terminal DNA-binding domain, and an N-terminal AHL-binding Domain. The activated AHL-LuxR complex binds to the promoters of quorum sensing regulated genes and modulates their expression. Often, the target genes also include the gene encoding the LuxI which create a positive feedback circuit within the quorum sensor (Parsek and Greenberg 2000). A typical Gram negative bacterial quorum sensing circuit is shown in Figure 1. More than 100 Gram negative bacterial species use quorum sensing for regulation of various processes and LuxI/LuxR homologs have been identified in most of them (Case et al. 2008).

AHLs have a conserved homoserine lactone (HSL) ring with a variable acyl side chain. AHLs are of two types (i) short-chain length having 4-8 carbon atoms in the acyl moiety and (ii) long-chain length having 10-18 carbon atoms. Acyl chains ranging from C4 to C18 have been identified with modifications such as carbonyl and hydroxyl moieties at the C3 position (Fuqua et al. 2001; Ng and Bassler 2009). Structure of different AHLs is shown in Figure 2. There is a wide chemical diversity in AHLs; still it promotes specific intra-species cell–cell communication in bacteria due to the high specificity of partner LuxR homologs proteins. Various LuxI homologs have substrate binding pockets of different sizes and shapes that accommodate only a particular acyl-ACP for synthesis of a particular type of AHL (Watson et al. 2002; Gould et al. 2004). Likewise, the AHL-detecting LuxR homologs have a unique binding pocket that specifically binds particular AHL ligands (Vannini et al. 2002; Zhang et al. 2002; Yao et al. 2006; Bottomley et al. 2007; Chen et al. 2011).

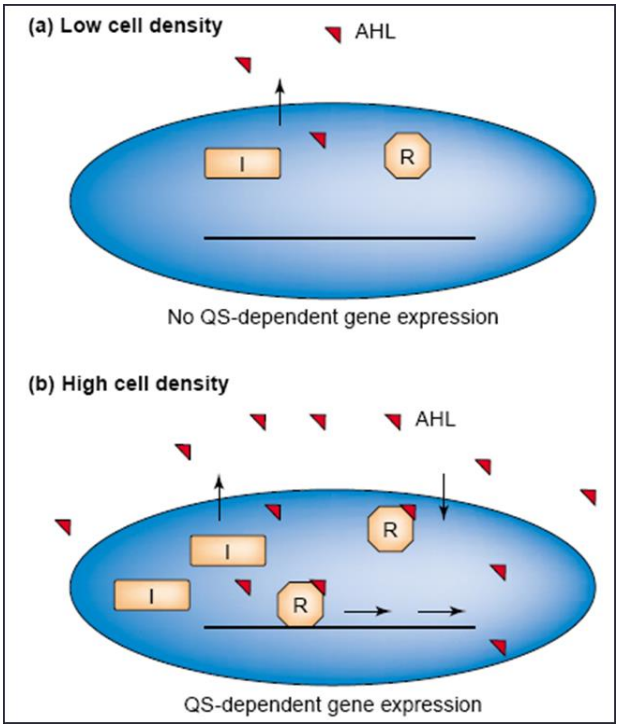

Figure 1 A quorum sensing model based on acyl homoserine lactone signalling systems

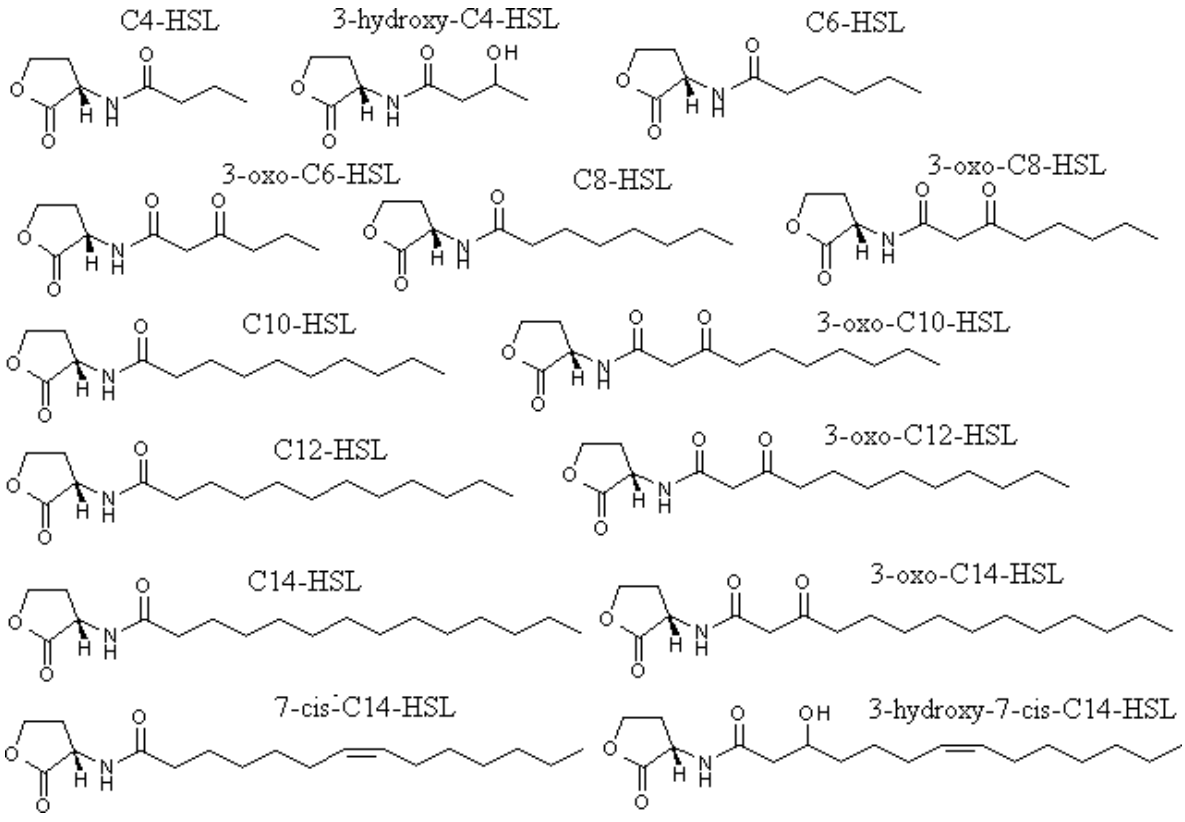

Figure 2 Structure of some representative AHLs

Numerous Gram-negative pathogens control virulence factor production using LuxI/LuxR type quorum sensing circuits (Table 1). We describe the *Pectobacterium carotovorum* and *Pseudomonas aeruginosa* quorum sensing systems as the canonical examples here.

Table 1 Diversity of quorum sensing circuits and quorum sensing regulated functions in various bacteria

Organism	Major signal molecule	Regulatory proteins	Phenotype
Vibrio fischeri	3-Oxo-C6-HSL	LuxI/LuxR	Bioluminescence
Vibrio harveyi	3-Hydroxy-C4-HSL	LuxLM/LuxN	Bioluminescence
Pseudomonas aeruginosa	3-Oxo-C12-HSL C4-HSL	LasI/LasR RhlI/RhlR	Multiple extracellular enzymes, RhlR, Xep, biofilm formation Multiple extracellular enzymes, rhamnolipid, RpoS, secondary metabolites
Pseudomonas aureofaciens	C6-HSL	PhzI/PhzR	Phenazine antibiotics
Agrobacterium tumefaciens	3-Oxo-C8-HSL	TraI/TraR	Ti plasmid conjugation
Erwinia carotovora subsp. carotovora	3-Oxo-C6-HSL	ExpI/ExpR CarI/CarR	Exoenzymes
Erwinia chrysanthemi	3-Oxo-C6-HSL	ExpI/ExpR	Carbapenem antibiotics
Erwinia stewartii	3-Oxo-C6-HSL	EsaI/EsaR	Pectate lyases
Rhizobium leguminosarum	C6-HSL C8-HSL 3-Hydroxy-7-cis-C14-HSL	RhiI/RhiR	Exopolysaccharide, virulence factors
Rhizobium etli	Not identified	RaiI/RaiR	Restriction of number of nitrogen-fixing nodules
Chromobacterium violaceum	C6-HSL	CviI/CviR	Exoenzymes, antibiotics, cyanide, violacein
Burkholderia cepacia	C8-HSL	CepI/R	Protease, siderophores
Aeromonas hydrophila	C4-HSL	AhyI/AhyR	Exoprotease production
Aeromonas salmonicida	C4-HSL	AsaI/AsaR	Extracellular protease
Ralstonia solanacearum	C8-HSL	SolI/SolR	Not identified
Serratia liquifaciens	C4-HSL	SwrI/SwrR	Extracellular protease, swarming

Rhodobacter sphaeroides	7-cis-C14-HSL	CerI/CerR	Dispersal from bacterial aggregates
Enterobacter agglomerans	3-Oxo-C6-HSL	EagI/EagR	Not identified
Escherichia coli	Not identified	?/SdiA	Cell division, attachment and effacing lesion formation
Yersinia enterocolitica	C6-HSL	YenI/YenR	Swimming and swarming motility
Yersinia pseudotuberculosis	C8-HSL	YesI/YesR	Motility and clumping

1.1.1 Quorum sensing regulated pathogenesis of *Pectobacterium carotovorum* subsp. *carotovorum* (*Pcc*)

1.1.1.1 Soft rot caused by *Pectobacterium carotovorum* subsp. *carotovorum* (*Pcc*)

Pectobacterium carotovorum subsp. *carotovorum* (*Pcc*) is one among the *Pectobacteria* or soft rot bacteria having the widest of host range, potato being the most important crop affected in temperate regions (Pérombelon 2002; Toth et al. 2003). Soft rot of potato tubers caused by *P. carotovorum* can result in extensive crop losses even post-harvest during storage (Perombelon and Kelman 1980; Pérombelon 2002). Soft rot affects several important crop and ornamental species across the world. Tuber soft rot is initiated under wet conditions at lenticels, the stolon end and/or in wounds. In storage, the lesion covers the whole tuber and subsequently spreads to neighbouring tubers. Tuber tissue is macerated and turns cream coloured which in the presence of air turns black, emanating foul smell after secondary organisms cause superinfection. Typical symptoms of soft rot caused by *Pcc* is shown is Figure 3 (a) As the seed tubers start rotting in the field blanking occurs before emergence. In insufficiently ventilated cold storage liquid from the rotting tuber can spread it to the neighbouring tubers due to spillage, creating massive rotting pockets in the stored tuber lot (Czajkowski et al. 2011). The progression of symptoms depends on the virulence of the bacterial strain and host plant susceptibility.

The initiation of soft rot disease is highly dependent on environmental conditions as the soft rot bacteria are considered as opportunistic pathogens (Pérombelon 2002). They live within the plant tissue without causing symptoms and this asymptomatic stage ends when high moisture and low oxygen concentration lower plant resistance which favors bacterial growth (Perombelon and Kelman 1980; Pérombelon 2002). *Pcc*, a typical necrotroph also displays a

broad host range. *Pcc* is also described as brute-force pathogen because its virulence strategy relies on necrotrophic mode of action where the plant cell wall degrading enzymes (PCWDE) physically attack the plant cell walls and disrupt the host cell integrity and thus promote rotting finally leading to death of the host cell tissue (Barras et al. 1994; Liu et al. 2008). Coordination of virulence factor synthesis is crucial for the pathogenicity which is carried out by quorum sensing (QS) through a complex set of transcription factors and posttranscriptional regulators (Chatterjee et al. 1995; Mukherjee et al. 2000; Hyytiäinen et al. 2001; Hyytiäinen et al. 2003; Kõiv et al. 2001).

1.1.1.2 ExpI/ExpR Quorum Sensing in *Pectobacterium carotovorum* subsp. *carotovorum*

Pcc has been divided in two classes depending on the type of AHL it produces. Class I strains produce N-3-oxooctanoyl-L-homoserine lactone (3-Oxo-C8HSL) and a low amount of 3-oxohexanoyl-L-homoserine lactone (3-Oxo-C6HSL). In contrast, class II strains produce predominantly 3-Oxo-C6HSL, whereas little or none of 3-Oxo-C8-HSL (Watson et al. 2002). In *Pcc*, the quorum sensing systems are complex in terms of regulation. Quorum sensing in *Pcc* is responsible for regulation of the Type three secretion system (T3SS), plant cell wall degrading enzymes (PCWDE) and antibiotic production. *Pcc* quorum sensing systems include up to three transcription activators capable of responding to two different AHL molecules that, in turn, are encoded by one synthase. The LuxI synthase homolog, ExpI (also referred to as CarI, AhlI and HslI), can synthesize 3-Oxo-C6HSL or 3-Oxo-C8HSL (Pirhonen et al. 1993). Once AHLs accumulate, they can interact with CarR, ExpR1 or ExpR2. These three are the LuxR homologs in *Pcc*. The interaction of AHL with CarR regulator is most straightforward. As shown in Figure 3(b), CarR binds 3-oxo-C6HSL and then binds the *carA* promoter, which controls the car operon that encodes the carbapenem antibiotic (Welch et al. 2000). The *car* operon is also controlled by the transcription regulator Hor, for which the mechanism is still unknown. The other two LuxR homologs, ExpR1 (also known as ExpR and EccR) and ExpR2 (also known as VirR), upregulate *rsmA* at sub-threshold levels of AHLs and directly inhibit PCWDE production (Sjoblom et al. 2006). RsmA belongs to the post-transcriptional Rsm system which is responsible for destabilization of mRNA transcripts that encode PCWDEs that includes cellulase, pectate lyase and protease. The Rsm system also includes *rsmB* that binds and down regulates RsmA, allowing translation of RsmA-targeted mRNAs. At threshold levels, 3-Oxo-C8HSL binds to ExpR1, whereas 3-oxo-C6HSL binds to ExpR2. When ExpR1 and ExpR2 bind to their cognate AHLs, they inhibit the expression of *rsmA* as a result, the mRNA transcripts that encode PCWDEs become free

(Sjoblom et al. 2006; Cui et al. 2005). A two component system of ExpS (sensor kinase) and ExpA (response regulator) regulates the transcription of *rsmB* (Eriksson et al. 1998; Cui et al. 2000). ExpS and ExpA appear to be homologues of GacS and GacA (or LemA and GacA) of number of Gram-negative bacteria (Heeb and Haas 2001). Thus, *Pcc* quorum sensing coordinates the virulence for successful infection through synergistic negative regulation of ExpR1 and ExpR2.

The next level of complexity occurs because expression of many of the PCWDEs is regulated positively by the breakdown products of plant cell wall produced by the activity of the bacterial pectinases on the tissues of plants. These products include 5-keto-4-deoxouronate, 2,5-diketo-3-deoxygluconate and 2-keto-3-deoxygluconate (Chatterjee et al. 1995; Nasser et al. 1994). In the presence of these metabolites regulation arises due to dissociation of the transcriptional repressor KdgR (Liu et al.1999). Binding sites for the KdgR repressor exist not only in the operators of many of the PCWDEs but also in *rsmB*. As a consequence the initial production of pectinases causes further induction of virulence genes at both transcriptionally as well as and post-transcriptionally (Hyytiainen et al. 2001). This has been represented in Figure 3(c). Breakdown products of the substrates released by the action of the bacterial PCWDEs also perform the role of signalling molecules for the plant, heralding the presence of a pathogen that triggers the hypersensitive disease response. The quorum sensing places pathogenicity associated genes under density dependent control which avoid the activation of host plant's defence systems (Newton and Fray 2004). Successful infection of *Pcc* requires a relatively high inoculum (Perombelon 2002), and the progression of the disease is then a competition between the development of plant resistance and bacterial multiplication (Perombelon and Kelman, 1980). Thus, the production of PCWDEs prematurely when the cell densities are low would give rise to an unsuccessful infection and on the contrary would induce local and systemic plant defence response, which in turn would resist subsequent infections. Thus, *Pcc* uses AHLs to monitor its cell density and only initiates a pathogenic attack when its population density is above a critical level, which ensures a high probability of overcoming host resistance (Mole et al. 2007; Põllumaa et al. 2012).

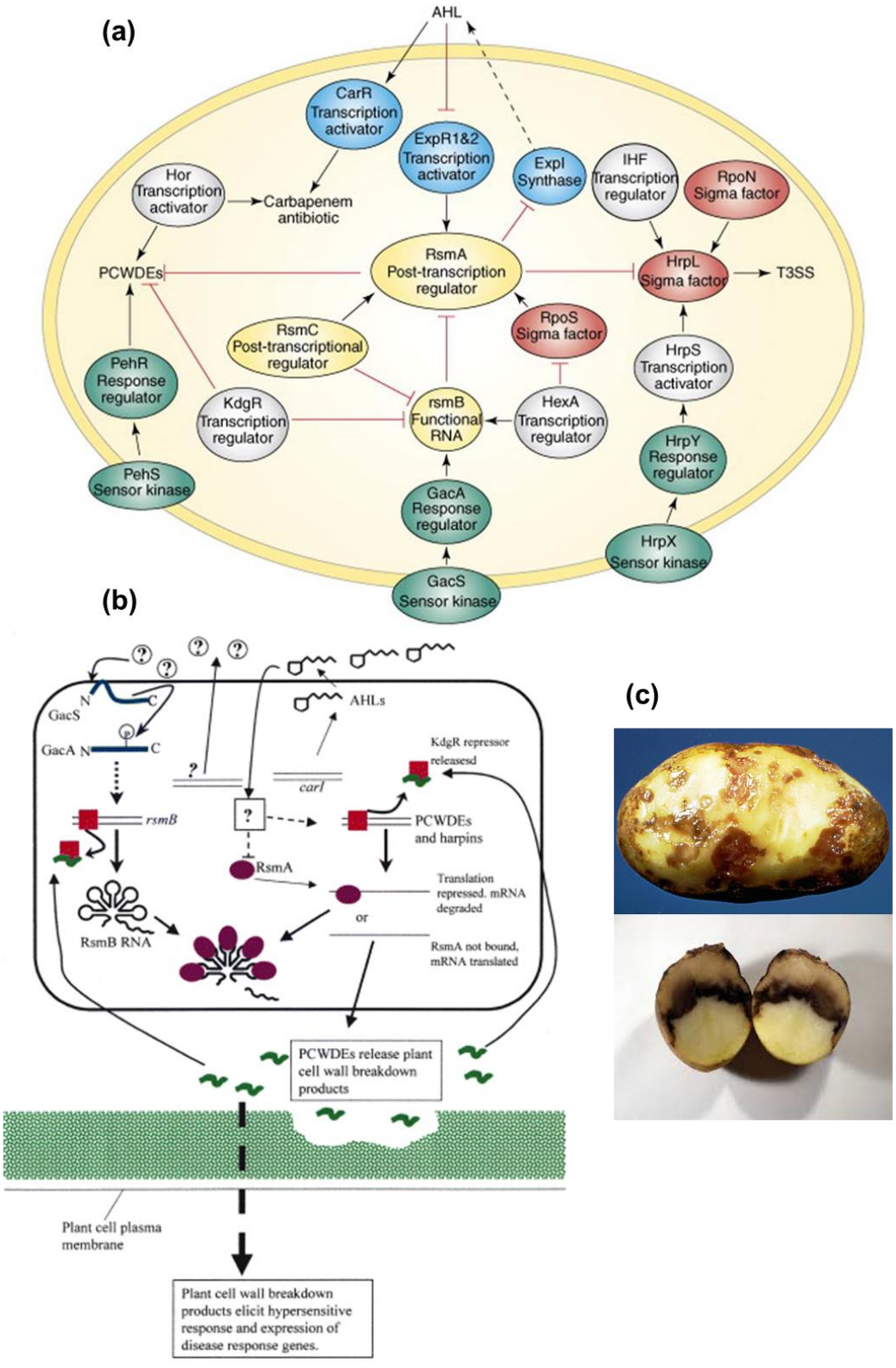

Figure 3 Soft rot caused due to quorum sensing in *Pectobacterium carotovorum* subsp. *carotovorum*

(a) Quorum sensing regulated production of antibiotic Carbapenem **(b)** Overview of quorum sensing regulated virulence in *Pcc* **(c)** Typical soft rot symptoms on potato

1.1.2 Quorum sensing regulated pathogenesis of *Pseudomonas aeruginosa*

P. aeruginosa can be isolated from diverse habitats including water, soil, animals, and plants (Parsek and Greenberg 2000; Rahme et al. 2000). In humans, it is the leading cause of bronchopulmonary infections in cystic fibrosis patients and nosocomial infections in burn victims and immunocompromised individuals (Fuqua and Greenberg 1998; Pesci and Iglewski 1997). In many plants, it is an opportunistic pathogen that causes soft rot. Infiltration of *P. aeruginosa* into *Arabidopsis* or into lettuce leaves causes initially water-soaked lesions and then chlorosis, followed by tissue maceration and systemic infection (Rahme et al. 2000). One of the reasons *P. aeruginosa* is a successful opportunistic pathogen and can infect diverse hosts is that it produces a battery of secreted virulence factors. These virulence factors include siderophores, exoproteases, lipases and exotoxins which are regulated by quorum sensing. Thus, even if *P. aeruginosa* caused plant infections are not a major reason for economic loss in agriculture, understanding about quorum sensing regulated pathogenesis of *P. aeruginosa* deserves a great attention because *P. aeruginosa* is not only a well-studied model for quorum sensing, but it has also been used in studies to define universal virulence mechanisms across phylogenetic boundaries. Usage of various mutants which were defective in causing virulence in different hosts such as mouse, *Arabidopsis*, lettuce, nematodes, and insects resulted in identification of genes which were regulated by quorum sensing and were necessary for full virulence in all hosts (Rahme et al. 2000; Tan 2002). There are advantages to *P. aeruginosa* in quorum sensing regulated virulence factors production for infecting any host. Production of extracellular virulence factors only after achieving a critical population density is favourable as a mass of cells is required to produce sufficient quantities of these factors to cause successful infection. The other advantage is the fact that *P. aeruginosa* can proliferate without producing virulence factors till it achieves a threshold population density and then the coordinated and simultaneous bulk production of virulence factors by the population can make the infection successful (Parsek and Greenberg 2000).

1.1.2.1 LasI/LasR and RhlI/RhlR quorum sensing in *Pseudomonas aeruginosa*

P. aeruginosa uses a dense network of quorum sensing receptors and regulators. There are four recognized quorum sensing pathways in *P. aeruginosa*: two LuxR and LuxI-type

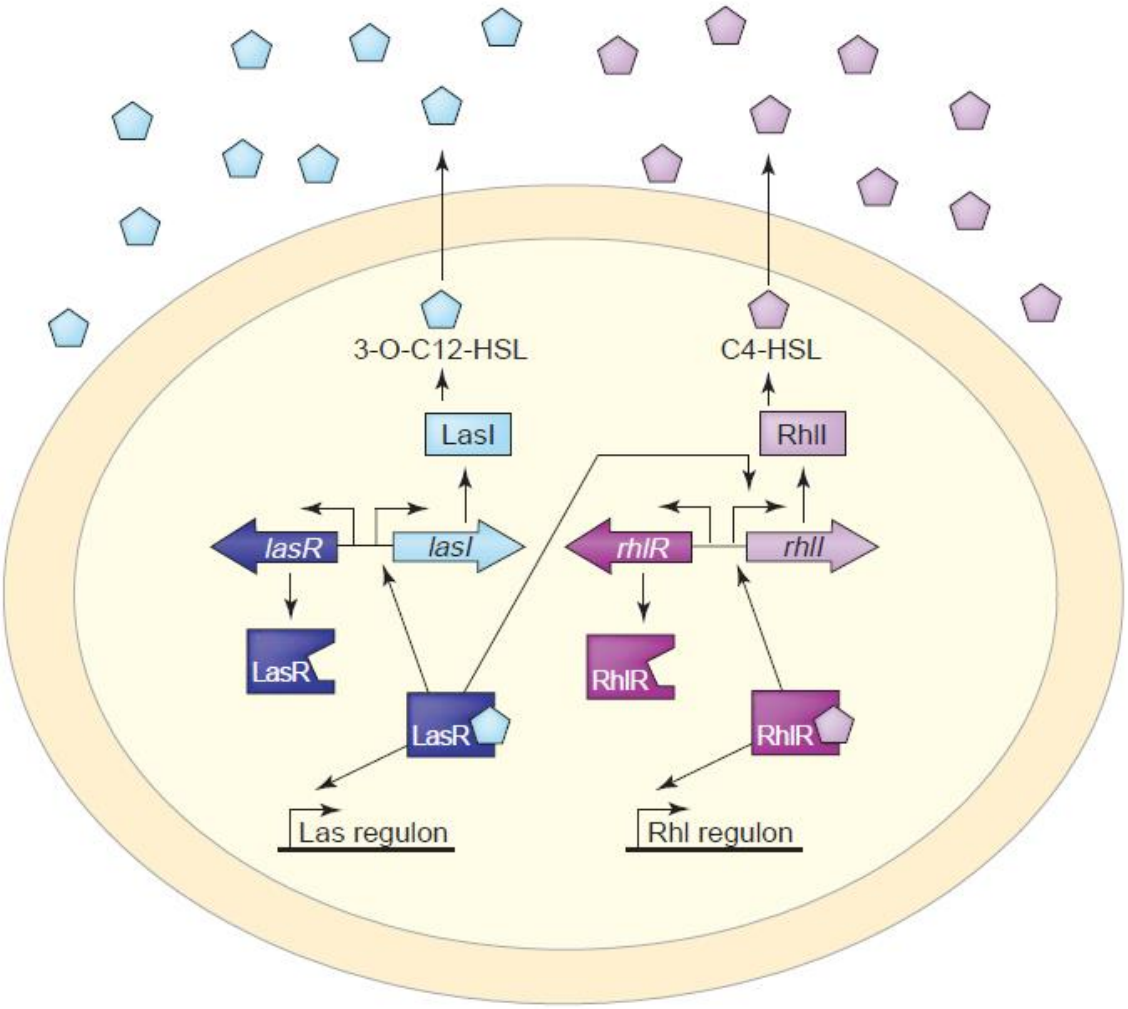

Figure 4 LasI/LasR and RhlI/RhlR quorum sensing in *Pseudomonas aeruginosa*

systems called LasR and LasI and RhlR and RhlI, the PqsR-controlled quinolone system and the IQS system. It has been reported that at least 6% (over 300 genes) of the *P. aeruginosa* genome is AHL-regulated via the *las* and *rhl* quorum sensing systems (Hentzer et al. 2003; Schuster et al. 2003; Wagner et al. 2004). The *las* and the *rhl* systems are organized hierarchically such that the *las* system exerts transcriptional control over both *rhlR* and *rhlI* (Latifi et al. 1996). The LasI synthesizes 3-oxo-C12-HSL (More et al. 1996; Val and Cronan 1998; Parsek et al. 1999; Gould et al. 2004; Bottomley et al. 2007). At low population densities LasI synthesizes a basal level of 3-oxo-C12-HSL. As density increases, 3-oxo-C12-HSL reaches to a critical concentration and interacts with LasR. The LasR–3-oxo-C12-HSL complex activates transcription of target genes which include virulence factors such as elastase, proteases, and exotoxin A (Gambello and Iglewski 1991; Gambello et al. 1993; Schuster et al. 2003, 2004). LasR–3-oxo-C12-HSL also activates *lasI* creating an autoinducing feed-forward loop (Seed et al. 1995). Another target of regulation by LasR–3-

oxo-C12-HSL is *rhlI* (Latifi et al. 1996; Pesci et al. 1997) which is responsible for the synthesis of butanoyl homoserine lactone (C4-HSL) (Ochsner et al. 1994; Pearson et al. 1995). At high concentrations, C4-HSL binds to RhlR and activates target genes, including those encoding elastase, siderophores, pyocyanin, and proteases (Schuster et al. 2003; Schuster and Greenberg 2007). This complex also creates an autoinduction loop by activating *rhlI*. *P. aeruginosa* quorum sensing activated virulence factors that include lectin, swarming motility, rhamnolipids, elastase, proteases, pyocyanin, and toxins. This is illustrated in Figure 4. LasR–3-oxo-C12-HSL and RhlR–C4-HSL activated gene regulation is not independent rather there is an extensive overlap between them. Another quorum sensing controlled phenotype in *P. aeruginosa* is biofilm formation. Regulation of biofilm formation in *P. aeruginosa* largely depends on additional environmental signals but quorum sensing regulation of rhamnolipids, swarming motility, and siderophores also contribute significantly to *P. aeruginosa* biofilm formation (Ochsner et al. 1994; Deziel et al. 2003; De Kievit 2009; Patriquin et al. 2008; Rahman et al. 2010; Rutherford and Bassler 2012).

1.2 Quorum quenching

Any process that can effectively interfere with quorum sensing is identified as quorum quenching (QQ) (Dong et al. 2001). As explained in above section (1.1) any of the three steps of AHL based quorum sensing regulation mechanism could be targets for quorum quenching procedures. The targets could be (1) the production of signal molecules (LuxI homologs), (2) sensing of the signal molecule by the cognate regulatory protein (AHL-LuxR), and (3) the signal molecule itself (AHLs). QQ mechanisms could be either of abiotic or biotic origins.

1.2.1 Signal synthesis (LuxI) as target for quorum quenching

Interfering with AHL synthesis is one of the approaches for the inhibition of quorum sensing. If no AHL is produced, no quorum sensing occurs. AHL synthesis progresses from S-adenosyl methionine (SAM) and a fatty acid, linked to an acyl carrier protein. It was found that several analogs of SAM inhibit the LuxI reaction (Parsek et al. 1999). The bactericidal molecule triclosan which targets the enoyl-acyl carrier protein reductase FabI, also affects the synthesis of AHL (Hoang and Schweizer 1999). This type of compound affects key metabolic compounds in bacteria. It therefore may affect functions other than those regulated by quorum sensing which can be a major drawback as anti-virulence strategy. In one of the recent study it was found that C8-HSL analog binds to AHL synthase and inhibits its activity. This compound significantly inhibited the production of C8-HSL in *Burkholderia glumae* in a

dose dependent manner by inhibiting its synthase (Chung et al. 2011). In another study a compound was identified which targets the recycling of SAM and inhibits AHL synthesis (Gutierrez et al. 2009).

1.2.2 Sensing of signal molecule (AHL-LuxR) as target for quorum quenching

The binding of AHL to LuxR protein is a crucial step in quorum sensing. Therefore antagonists that interfere with binding of AHL with the receptor are potential quorum sensing inhibitors. Various natural and synthetic compounds have been identified as inhibitors. In general, the analogs of AHL are potential inhibitors of native AHL. Analogs with differences in the acyl side chain of 3-oxo-C6-HSL for *Vibrio fischeri*, 3-oxo-C12-HSL for *Pseudomonas aeruginosa* and 3-oxo-C8-HSL for *Agrobacterium tumefaciens* were established to inhibit the binding of native AHLs (Passador et al. 1996; Schaefer et al. 1996; Zhu et al. 1998). Furthermore, a library of synthetic analogs to the *Pseudomonas aeruginosa las* quorum sensing molecule 3- oxo-C12-HSL was constructed by Smith et al. (2003). From this library three compounds acted as antagonists against 3-oxo-C12-HSL-LasR mediated quorum sensing. Some natural compounds have also been identified as inhibitors. Halogenated acyl-furanones, having similar structure to AHLs, which are acquired from the marine algae *Delisea pulchra* are the most studied inhibitors (Givskov et al. 1996). These compounds displace the 3-oxo-C6-HSL from its cognate LuxR receptor and inhibit the quorum sensing mediated gene expression (Manefield et al. 1999). In addition, other natural AHL inhibitors have been identified, such as patulin and penicillic acid which inhibit *Pseudomonas aeruginosa* quorum sensing of either or both the *las* and *rhl* systems (Rasmussen et al. 2005; Jakobsen et al. 2012; Jakobsen et al. 2012).

1.2.3 AHL as target for quorum quenching

Along with small molecules which interfere with signal sensing or generation, signal degradation by catalytic enzymes is a substitute strategy. Figure 5 depicts the diversity of quorum quenching enzymes and inhibitors. The first reports of an enzymatic degradation of AHL came from soil bacterial isolates of *Variovorax* and *Bacillus* genera (Dong et al. 2000; Leadbetter and Greenberg 2000). From then on, several enzymes which can degrade or modify AHL have been reported. There are four groups of enzymes with different catalytic mechanisms involved:

(1) The reductases which convert 3-oxo-substituted AHL to their cognate 3-hydroxyl substituted AHL and

(2) The oxidases that catalyze oxidation of the acyl chain
(3) The acylases (also referred to as amidohydrolases or amidases) that cleave AHLs at the amide bond and release fatty acid and homoserine lactone,
(4) The lactonases that open the homoserine lactone ring.

They occur in bacteria, archaea and eukaryotes (Grandclement et al. 2016). The only known quorum quenching reductase is BpiB09 from a metagenomic library (Bijtenhoorn et al. 2011). BpiB09 belong to the short-chain reductase family, the members of which comprise a dinucleotide cofactor-binding site. The cytochrome P450 oxidase CYP102A1 of *B. megaterium* is reported to oxidise the ω-1, ω-2 and ω-3 C atoms of acyl chain of AHL (Chowdhary et al. 2007). AHL acylase and AHL lactonase are the two groups of enzymes which are predominantly reported from bacterial origin.

1.2.3.1 AHL acylase

A major group of AHL degrading enzymes, acylases, cleave the amide bond which connects the fatty acyl chain to the homoserine lactone. The first AHL acylase was discovered by Leadbetter and Greenberg (2000), from *Variovorax paradoxus* which could utilize AHLs as a sole source of energy and nitrogen. It was further observed that this strain was using acyl group of different AHLs as energy source and not the lactone ring. Similar behaviour was also observed for *R. erythropolis*, which possesses three types of AHL inactivating and degrading enzymes (Uroz et al. 2005). The AHL acylase identified from *Ralstonia* strain XJ12B shared amino acid sequence homology with members of the N- terminal nucleophile hydrolase (Ntn-hydrolase) superfamily. Since the discovery of this enzyme the family of AHL acylases showing similarity with the acylase of *Ralstonia* was named as AiiD. Heterologous expression of *aiiD* in *P. aeruginosa* inhibited the AHL accumulation in the culture medium, decreased virulence, and impaired swarming motility as well as attenuated paralysis and killing of *C. elegans*. There are several homologues of AiiD having 38 to 83% amino acid sequence overlap which was found out by sequence alignment against genome databases that may encode for similar AHL acylases. These homologues were found in the

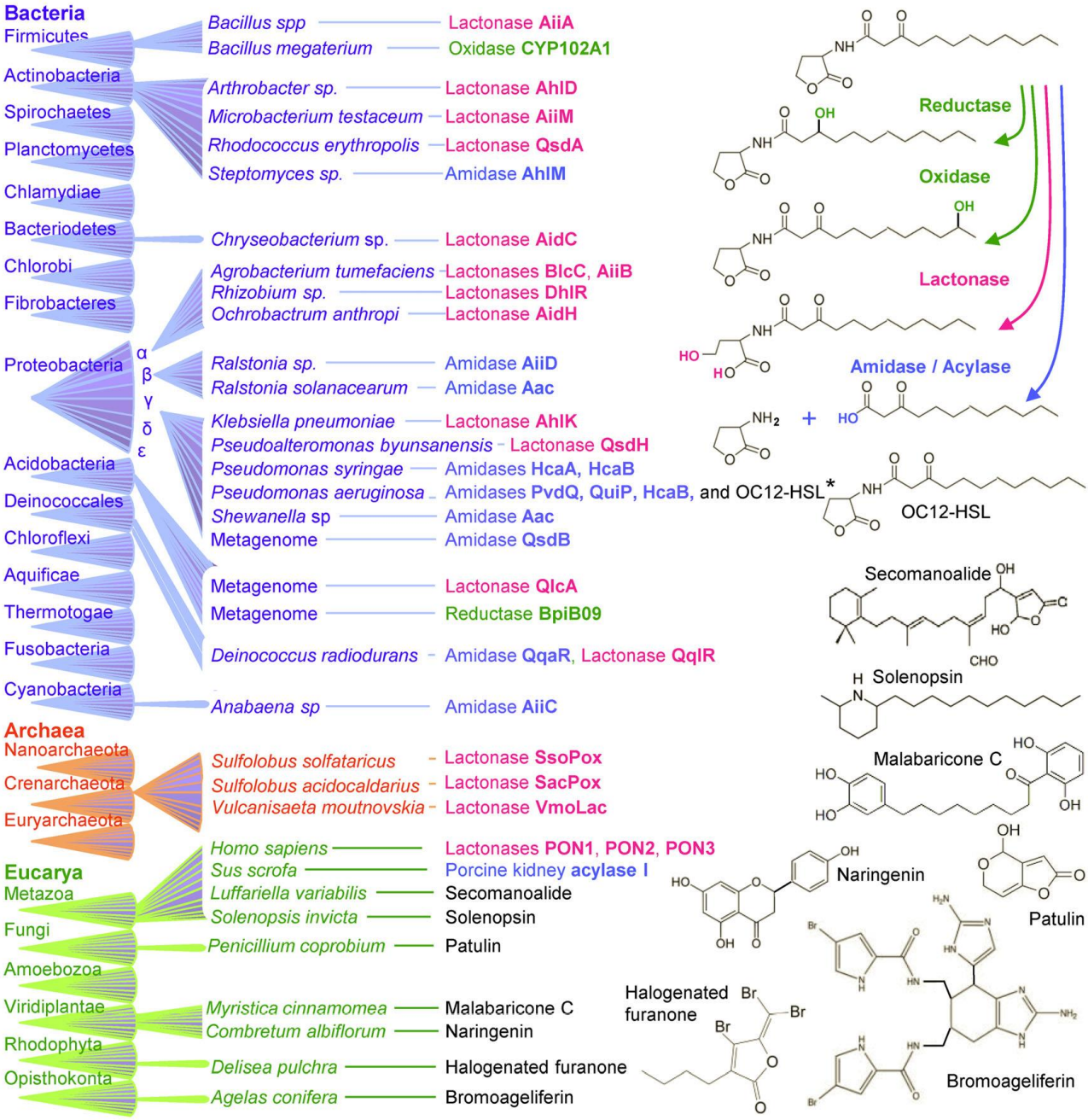

Figure 5 Diversity of quorum quenching enzymes and inhibitors in different species of bacteria, Archaea and Eukaryote

genome of R. solanacearum, R. metallidurans, P. fluorescence, P. putida, P. aeruginosa, P. syringae and Deinococcus radiodurans (Lin et al. 2003).

Two strains of *P. aeruginosa* were found out growing on AHLs as sole source of carbon but these isolates utilized only long chain AHLs having acyl chain longer than 8 carbons (Huang et al. 2003). An AHL acylase called PvdQ was responsible for this activity in *P. aeruginosa*

strains. When *pvdQ* was overexpressed in *P. aeruginosa* PAO1 it resulted in decreased accumulation of 3-oxo-C12-HSL by *P. aeruginosa* PAO1. A second AHL acylase was also found form *P. aeruginosa* named QuiP which was able to utilize and inactivate quorum sensing signals (Huang et al. 2006). QuiP shares 21% amino acid sequence homology with PvdQ and 23% homology with AiiD from *Ralstonia* spp. PvdQ and QuiP both belong to Ntn-hydrolase family.

An AHL acylase was also found from Gram-positive *Streptomyces* sp. M664 this suggested that AHL acylases are ubiquitous among diverse bacterial genera. The AHL acylase form *Streptomyces* sp. is known as AlhM, a member of the Ntn- hydrolase superfamily. AlhM shares 35% and 32% amino acid sequence homology with AiiD from *Ralstonia* strain XJ12B and PvdQ from *P. aeruginosa* PAO1, respectively. The catalytic activity of AlhM is higher for long chain AHLs having more than 8 carbons in acyl chain. Upon addition of purified AlhM in the culture of *P. aeruginosa* there was decreased accumulation of 3-oxo-C12-HSL in the culture medium and further reduction in the virulence (Park et al. 2005).

Another acylase called AiiC, was identified from *Anabaena* sp. strain *PCC*7120 (Romero et al. 2008). This enzyme shared 29% amino acid sequence homology with QuiP of *P. aeruginosa* and also belongs to the Ntn-hydrolase family. AiiC has the ability to hydrolyze AHLs with 4-16 carbons in the acyl chain, with or without the 3-oxo moiety. A different AHL acylase was found in *Comamonas* strain D1 showing wide substrate specificity for different AHLs, but surprisingly *Comamonas* reportedly could not grow on AHLs as a sole source of carbon or nitrogen (Uroz et al. 2007). A novel AHL acylase belonging to *α*/*β*-hydrolase fold protein from *Delftia* sp. VM4 was purified and its kinetics and thermodynamics parameters were studied in detail (Maisuria and Nerurkar 2015). Different AHL acylases known form various bacteria are listed in Table 2.

Table 2 AHL acylases in various bacteria

AHL acylase	Host	Substrate
AiiD	*Ralstonia eutropia*	C8-12-HSL
PvdQ	*Psedomonas aeruginosa*	C7-12-HSL with or without C3-substitution
QuiP	*Psedomonas aeruginosa*	C7-14-HSL with or without C3-substitution
AiiC	*Anabaena sp. PCC 7120*	Chain length more than C10

AhlM	*Streptomyces sp. M664*	Chain length more than C8
	Ralstonia solanacearum	Chain length more than C6
Aac	*Shewanella sp. MIB015*	Broad but prefer long chain
	Pseudomonas syringae	C8,C10, C12-HSL
HacA	*Pseudomonas syringae*	C6-12-HSL with or without C3-substitution
HacB	*Variovorax sp.*	Broad
	Variovorax paradoxus	Broad
	Tenacibaculum maritimum	C10-HSL
	Comomonas sp. D1	C4-16-AHL with or without C3-substitution
	Rhodococcus erythropolis W2	C10-HSL
	Delftia sp. VM4	C6-HSL and 3-oxo-C6-HSL

1.2.3.2 AHL lactonase

AHL lactonases are enzymes that hydrolyze the lactone ring of the homoserine moiety of AHLs and do not affect the rest of the structure. Amino acid sequence and protein structure of the AHL lactonases are diverse. There are four lactonase families known: the metallo-*β*-lactamase-like lactonases, the phosphotriesterase-like lactonases, the paraoxonases and the *α*/*β*-hydrolase fold lactonases.

The metallo-*β*-lactamase-like lactonases have a unique fold displayed by AiiA from *Bacillus thuringiensis* and AiiB from *Agrobacterium tumefaciens* (Liu et al. 2005, 2007). This family consists other members such as AttM, AhlD and QlcA which were identified in cultured bacteria and metagenomes (Dong et al. 2000; Carlier et al. 2003; Park et al. 2003; Riaz et al. 2008). The first AHL inactivating enzyme was identified by Dong et al. (2000). When the authors screened treated soil samples and laboratory bacterial collections a Gram-positive *Bacillus* sp. 240B1 showed the phenotype of AHL inactivation. The gene (*aiiA*) encoding the enzyme was cloned from it and the gene product, a 250 amino acid protein, was characterized as a lactonase. There was no homology found to any of the known enzyme families, but the enzyme was characterized as a zinc metalloprotease based on a conserved short sequence of HXHXDH~60aa~H, which is known as a zinc-binding motif shared by several zinc-metalloproteases. Upon over-expression of *aiiA* in *Erwinia carotovora* it was observed that there was reduction in AHL accumulation in the culture which resulted in significant decrease in the secretion of extracellular pectolytic enzymes. Furthermore, when the strain

overexpressing *aiiA* was inoculated into Chinese cabbage, cauliflower, and tobacco plants, maceration of plant tissue was prevented whereas wild-type infected plants showed severe maceration (Dong et al. 2001, 2002). Shortly after this, several similar AiiA-like enzymes were identified from different *Bacillus* species which shared more than 90% sequence homology (Lee et al. 2002). A different AHL lactonase was identified from *A. tumefaciens* encoded by gene known as *aiiB* (Carlier et al. 2003) but AiiB shared only 28% homology with AiiA. AiiB showed preference for substrates with longer acyl chain. Further, from several species such as *Agrobacterium tumefaciens*, *Klebsiella pneumoniae* and *Arthrobacter* sp. AHL lactonases were identified which were designated as *attM*, *ahlK* and *ahlD* respectively. AHL lactonases belonging to this family have the conserved zinc-binding motif suggesting similarity in their catalytic mechanism.

The phosphotriesterase like lactonases family encompasses enzymes, such as QsdA and Vmo-Lac (Uroz et al. 2008; Hiblot et al. 2013). QsdA is 323 amino acid containing protein and was identified from *Rhodococcus erythropolis*. Phosphotriesterase are zinc-metalloproteases that hydrolyze phosphotriester which contain organophosphorus compounds, but in recent times more members of this family were found to possess lactonase and amidohydrolase activity as well (Uroz et al. 2008). A phylogenetic study established that QsdA homologues are abundant among the *Rhodococcus* genus which can hydrolyze AHLs ranging in acyl chain length from 6 to 14 carbons regardless of the oxidation state in the C3 carbon position.

The structure of a *α*/*β*-hydrolase fold lactonase identified as AidH and isolated from the bacterium *Ochrobactrum* has been determined (Gao et al. 2013) which does not demonstrate metal-binding motif HXHXDH. Hence, a novel catalytic mechanism was proposed which differs from that of other lactonases.

The paraoxonases are extensively studied in mammals and are not reported to have bacterial origin (Harel et al. 2004; Ben-David et al. 2012). The catalytic mechanisms of these lactonases differ in detail from above mentioned lactonases. Different AHL lactonases known form various bacteria are listed in Table 3.

Table 3 AHL lactonase in various bacteria

AHL lactonase	Host	Substrate
	Bacillus sp. 240B1	C6-10-HSL
	Bacillus cereus A24	AHL

AiiA	*Bacillus mycoides*	AHL
	Bacillus thuringiensis	AHL
	Bacillus anthracis	C6, C8, C10-HSL
AttM	*Agrobacterium tumefaciens*	3-oxo-C8-HSL, C6-HSL
AiiB	*Agrobacterium tumefaciens C58*	Broad
AiiS	*Agrobacterium radiobacter K84*	Broad
AhlD	*Arthrobacter sp. IBN110*	Broad
AhlK	*Klebsiella pneumonia*	C6-8-HSL
QlcA	*Acidobacteria*	C6-8-HSL
AiiM	*Microbacterium testaceum StLB037*	C6-10-HSL
QsdA	*Rhodococcus erthropolis W2*	C6-14-HSL with or without C3 substitution
AidH	*Ochrobactrum sp. T63*	C4-10-HSL
DlhR, QsdR1	*Rhizobium sp. NGR234*	Not detected
AhlS	*Solibacillus silvestris StLB046*	C6-HSL, C10-HSL
SsoPox	*Sulfolobus solfataricus strain P2*	C8-12-HSL
	Rhodococcus sp.	Broad
GKL	*Geobacillus kaustophilus strain HTA426*	C6-12-HSL
PPH	*Mycobacterium tubercolosis*	C4, C8, C10-HSL
MCP	*Mycobacterium avium subsp. paratuberculosis*	C7-12-HSL
BpiB01, BpiB04, BpiB05, BpiB07	Soil metagenome	3-oxo-C8-HSL
QlcA	Soil metagenome	C6-10-HSL

1.2.4 Substrate specificity of AHL degrading enzymes

The AHL degrading bacteria generally show some degree of substrate preference. The factors such as amino acid structure, and/or cofactor involvement of the AHL degrading enzymes and the type of AHLs produced by other bacteria in the natural communities in the

surrounding of the AHL degrading enzyme producing bacteria affect the preferences (Huang et al. 2003, 2006; Carlier et al. 2003; Lin et al. 2003).

Majority of AHL lactonases as they hydrolyse the homoserine lactone ring display a wide range of substrate specificity and can cleave AHLs with different length of the carbon acyl side chain (Wang et al. 2004; Dong and Zhang 2005). Some of the examples are the AiiA from *Bacillus* sp. (Dong et al. 2000; Wang et al. 2004; Lui et al. 2005; Kim et al. 2005) and AhlD lactonases and *Arthrobacter* sp. (Park et al. 2003), respectively. Among AHL acylases, there are rare examples of broad range substrate specificity. One of them is the AiiC acylase from *Anabaena* sp. which can hydrolyze a set of AHLs that differ in the acyl chain length and substitution, including C4-HSL; however, it shows preference for long-chain AHLs (Romero et al. 2008). The other AHL acylases such as QuiP and PvdQ from *P. aeruginosa* were found to degrade only 3-oxo-C12-HSL (Huang et al. 2003, 2006). Similarly AiiD, the AHL acylase produced by *R. eutropha* degrades only unsubstituted and 3-oxo-substituted AHLs with acyl side chains consisting of at least 8 carbons (Lin et al. 2003).

1.2.5 Occurrence of AHL degrading enzymes

Majority of the AHL degrading bacteria that are reported were isolated from soil or rhizosphere of plants (Lee et al. 2002; Huang et al. 2003; Wang et al. 2004; Park et al. 2005; Yoon et al. 2006). These niches which are occupied by AHL degrading bacteria demonstrate lot of differences in the availability of nutrients, water, vital ions, and oxygen. This kind of surrounding generally fails to create optimal conditions for the AHL degrading enzymes. For this reason probably in bacteria AHL degrading enzymes are produced predominantly intracellularly where they can get fairly constant, optimal reaction conditions for their activity. Both AHL lactonases and AHL acylases are reported to be cytoplasmic (Leadbetter and Greenberg 2000; Dong et al. 2000, 2001, 2002; Zhang et al. 2002; Carlier et al. 2003; Park et al. 2003; Huang et al. 2003; Lin et al. 2003; Molina et al. 2003; Uroz et al. 2003, 2005; Wang et al. 2004; Liu et al. 2005; Kim et al. 2005; Jafra et al. 2006). A rare example of extracellular AHL acylase is reported from *Streptomyces* sp. (Park et al. 2005). Huang et al. (2012) have reported another rare example of AHL lactonase found in marine bacteria *Pseudoalteromonas* wherein the enzyme is located in the inner membrane of the periplasmic space. Another Gram negative organism *Muricauda olearia* has also been reported with an extracellular AHL lactonase (Tang et al. 2015).

1.3 Quorum quenching as an anti-virulence strategy

Quorum Quenching strategies do not aim to kill bacteria or limit bacterial growth but affect the expression of a specific function. This exerts a limited selective pressure for microbial survival than biocide treatments. This is a valuable trait for the development of sustainable biocontrol or therapeutic procedures in the present context of rising antibiotic resistance (Uroz et al. 2009). Most bacterial plant pathogens as discussed in previous sections, such as *Pectobacterium carotovorum* and *Pseudomonas aeruginosa*, rely upon AHL mediated quorum sensing to turn on gene cascades for their key virulence factors. This suggests that disrupting quorum sensing regulation could be a valuable approach to develop new phytoprotective agents viz. plant pathogens that rely upon quorum sensing for the regulation of pathogenicity. Till date, quorums quenching based biocontrol approaches have been evaluated under laboratory conditions, and field assays are not reported in the literature. Several plants take up and respond to AHLs (Schuhegger et al. 2006; Palmer et al. 2014; Sieper et al. 2014), but only a few of these plants such as clover and birdfoot deervetch are known to exhibit AHL degrading activities (Delalande et al. 2005; Gotz et al. 2007). There are three main biocontrol strategies as described that have been proposed for the attenuation of bacterial infection development in plants (Czajkowski and Jafra 2009).

1.3.1 Expression of a gene coding AHL synthase in the plant tissue

As bacterial populations use signal molecules to sense cell density and coordinate their own behavior, the artificially increased level of AHLs, produced by this transgenic plant, makes bacteria to misinterpret the population size. Such misinterpretation leads to the production of virulence determinants long before the pathogen population is large enough to sustain infection in the plant. This allows the plant to turn on its defense mechanisms and eliminate the pathogens before they can establish the infection.

1.3.2 Heterologous expression of genes encoding AHL-degrading enzymes in pathogen cell or in plant tissue

Transgenic plants encoding the AHL degrading enzymes have been shown to successfully provide resistance to the plants against the phytopathogens. *Bacillus* AiiA and *Agrobacterium* AttM lactonases were expressed into different plants (Dong et al. 2001; Ban et al. 2009; Vanjildorj et al. 2009; D'Angelo-Picard et al. 2011). These plants either showed a lower level of symptoms or an absence of symptoms which were regulated by quorum sensing in

pathogens like *Pectobacterium*. Moreover, transgenic plants expressing the lactonase AttM was not found to alter diversity of the root-associated bacterial populations (D'Angelo-Picard et al. 2011), which suggests the minimal impact of such strategies on the dynamics of the plant microbiota.

Recombinant phytopathogens expressing the AHL degrading enzyme have also shown successful decrease in their virulence. Dong et al (2000) showed that the production of exoenzymes and disease symptoms is impeded by the heterologous expression of the aiiA gene encoding the AiiA lactonase from *Bacillus* sp. in *P. carotovorum* cells.

1.3.3 Employing natural AHL degrading bacteria as biocontrol agents

In consideration of the debate that exists about the use and release of genetically modified organisms**,** a more acceptable biocontrol approach would be to isolate bacteria, capable of degrading AHL, from the natural environment and employing them as biocontrol agents. These studies have been facilitated by the incidence of AHL degrading bacteria in rhizospheric environments. This community represents a sizable percentage of the culturable bacteria (Dong et al. 2000; Steidle et al. 2001; Morello et al. 2004; D'Angelo-Picard et al. 2004, 2005). *Bacillus* and *Rhodococcus* are such bacteria which are reported not to inhibit the growth of the *Pectobacterium carotovorum*, but degrade its AHL and abolish the soft rot symptoms (Uroz et al. 2003, 2008; Dong et al. 2004). The AHL lactonase mutant of *B. thuringiensis* neither could degrade the AHL produced nor could inhibit the soft rot produced by *P. carotovorum* (Dong et al. 2004). In *Rhodococcus erythropolis* there are multiple enzymes (lactonase, amidase, reductase) responsible for AHL degradation (Uroz et al. 2005, 2008) but only the *qsdA* gene, coding for a lactonase, has been characterized so far which is known to degrade *P. carotovorum* AHL and can attenuate soft rot (Uroz et al. 2008). Co-culture of an AHL degrading *Acinetobacter* strain C1010 with plant pathogens *Burkholderia glumae* and *E. carotovora*, the causative agent of rot disease, have been successful in reducing virulence and disease symptoms (Kang et al. 2004).

Biocontrol strategies which target quorum sensing of the plant pathogens may suffer with some limitations. In some *Pseudomonas* strains which are used as biocontrol agents, quorum sensing is reported to positively regulate the expression of plant growth-promoting functions, e.g. production of antibiotic and antifungal molecules against all fungal pathogens. In a study carried out by Molina et al. (2003) the incompatibility of the quorum quenching based

biocontrol agents and other plant growth promoting bacteria has been demonstrated. In this scenario the spatial structure and vicinity of the quorum quenching bacteria and the quorum sensing bacteria (pathogenic/beneficial) in the rhizosphere of host plant would play major role to achieve the balanced outcome of quorum quenching based biocontrol approach. Hence the appropriate use of quorum quenching biocontrol agents to get optimum and desirable effect needs further evaluations. Moreover, in order to communicate efficiently, the spatial structure of pathogen cells on the root of the host plant plays a key role. The distance travelled by an AHL from an AHL producing cell to an AHL perceiving cell (calling distance) is a factor which determines quorum sensing efficiency in natural environment (Gantner et al. 2006). To disrupt quorum sensing (prerequisite to cause infection), the quorum quenching biocontrol agent should be able to colonise roots in such a manner that it is able to stop signalling between the pathogen cells. Considering the AHL calling distance, and the biogeography of the pathogen and biocontrol agent on the plant root are important parameters which will determine the efficiency of quorum quenching as biocontrol strategy.

1.4 Biological control

Biological control refers to the utilization of introduced or resident living organisms, other than disease resistant host plants with the intention of suppressing the pathogenesis caused by one or more plant pathogens. Biocontrol agents, which are used for this are living organisms or natural products derived from these organisms. Biocontrol strategy shows several advantages when compared to chemical products. The biocontrol agents decompose more quickly in the environment, are less toxic towards non-target species and suppress resistance development in pathogens. Because of these properties biocontrol agents can be applied in alternation with other pesticides to avoid resistance development (Thakore 2006).

Microorganisms which grow in rhizosphere are most suitable for use as biocontrol agents, since the rhizosphere is the primary protection for roots against attack by pathogens. Pathogens restricted through antagonism exhibited by microorganisms residing in rhizosphere before and during primary infection and also during secondary spread on the root. A good biocontrol agent should have a good degree of persistence and aggressiveness but be non-pathogenic to the host. Antibiosis, enzymes, toxic products, interference, competition etc. are some of the types of biological control mechanisms (Pal et al. 2006). However, for the mechanisms like antibiosis, target bacteria can develop resistance gradually.

So any strategy that can effectively stop pathogenic infection, but does not impose selection pressure, would be a promising alternative against phytopathogenic microorganism.

As biological control can result from many different types of interactions between organisms, researchers have focused on characterizing the mechanisms operating in different experimental situations. All pathogens are antagonized by the presence and activities of other organisms that they encounter. The different mechanisms of antagonism are related to interspecies contact as well as specificity of the interactions. Biocontrol is often attributed to antibiosis. In many biocontrol systems that have been studied, antibiotics have been shown to play a role in disease suppression. Antibiotics are microbial toxins that can, at low concentrations, poison or kill other microorganisms. Most microbes produce and secrete compounds with antibiotic activity. In some cases, antibiotics produced by microbes were effective at inhibiting plant pathogens and the diseases they cause. A short list of antibiotics that are identified, includes amphisin, 2,4-diacetylphloroglucinol (DAPG), hydrogen cyanide, oomycin A, phenazine, pyoluteorin, pyrrolnitrin, tensin, tropolone, and cyclic lipopeptides produced by pseudomonads and oligomycin A, kanosamine, zwittermicin A, and xanthobaccin produced by *Bacillus*, *Streptomyces*, and *Stenotrophomonas* spp. Many micro-organisms secrete lytic enzymes which are capable of hydrolyzing a wide variety of polymeric compounds including chitin, proteins, cellulose, hemicellulose, and DNA. Expression and secretion of these enzymes by different microbes result in the suppression of activities of plant pathogens directly. For example, control of *Sclerotium rolfsii* by *Serratia marcescens* appeared to be mediated by chitinase expression. And, a β-1,3-glucanase contributes significantly to biocontrol activities of *Lysobacter enzymogenes* strain C3 (Pal et al. 2006). Another mechanism of biological control is the detoxification of pathogen virulence factors. For example, certain biocontrol agents are able to detoxify albicidin toxin produced by *Xanthomonas albilineans*. Hydrogen cyanide (HCN) effectively blocks the cytochrome oxidase pathway and is highly toxic to all aerobic microorganisms at picomolar concentrations. The HCN produced by certain fluorescent pseudomonads is involved in the suppression of root pathogens (Compant et al. 2005). The root surface and surrounding rhizosphere are significant carbon sinks. Photosynthate allocation to this zone can be as high as 40%. Thus, along root surfaces there are various suitable nutrient rich niches which attract diverse microorganisms, and for the same reason phytopathogens are also attracted to these niches. Competition for these nutrients and niches can a key mechanism by which biocontrol agents can protect plants from phytopathogens. Some root exudates can also be effective as

antimicrobial agents (Compant et al. 2005). Plants also respond to a variety of chemical stimuli produced by soil- and plant-associated microbes. Such stimuli can also induce plant host defences and develop resistance against subsequent infection by a variety of pathogens. Induction of host defences can be local and/or systemic in nature, depending on the type of stimuli (Pal et al. 2006).

The biocontrol strains mainly belong to *Bacillus* and *Pseudomonas* bacterial genera. Most biocontrol studies are targeted at ornamental and food crop plants grown in greenhouses and only a few of them have reached to the field scale. The first studies on potato plant biocontrol focused on fighting against different *Pectobacterium* species (P´erombelon 2002; Toth et al. 2006). In these studies the fluorescent pseudomonads which were selected for their antagonistic activity in *in vitro* experiments were used and the ability to colonize and the persistence of *Pseudomonas* strains on treated plants were assessed (Kloepper and Schroth 1981; Kloepper 1983). The biocontrol mechanism of these strains were identified as 2,4 diacetylphloroglucinol antibiotic synthesis, iron competition via pyoverdine and pseudobactin production and their related receptors (DeWeger et al. 1986; 1995; Xu and Gross 1986; Rhodes and Logan 1987; Cronin et al. 1997). Moreover, the influence of external environment such as soil texture and pH promoting the expression of protecting activities was also studied. However, large-scale application of these biocontrol *Pseudomonas* showed limited effect. Gross (1988) demonstrated that selected strains were able to colonize plants, but were ineffective in *Pectobacterium atrosepticum* disease suppression. Members of *Bacillus* spp. because of their effective biocontrol activities such as endospore production and consequent resistance of these bacteria to environmental stresses drew the attention of researchers (Jacobsen et al. 2004). Therefore, *Bacillus subtilis* strains were tested for the control of potato diseases caused by *Pectobacterium* spp. and revealed reduced maceration symptoms in planta (Sharga and Lyon 1998). Since then, many other experiments have been conducted with *Bacillus* and *Pseudomonas* agents on potato pathogens (Table 4). The selected strains showed antibiotic activity against pathogens (*in vitro* screening), a strong ability to colonize roots (soil microcosm) or to induce systemic resistance of plant. More biocontrol agents are being isolated from bacterial species mainly which colonise in the rhizosphere because they generally demonstrate the ability to reside within the same ecological niche as the pathogen and results in a competition which shows the potential to eliminate pathogens (Neeno-Eckwall et al. 2001; Krechel et al. 2002; Sessitsch et al. 2002; Hiltunen et al. 2009). Hyper parasitism and the induction of potato defences are also some

examples of different biocontrol approaches demonstrated by antagonistic fungi for inhibiting Rhizoctonia black scurf and Verticillium wilt (Table 4).

Many different types of methods are tested by researchers for studying the potentials of the biocontrol agents in controlling the diseases caused by pathogens on host plants, before they can be finally used at field level. Pathogens enter and inhabit different tissues of plant and cause various diseases. Using different plant parts in *in vitro* assay to study effect of biocontrol agent gives more insight before studying the effectiveness of the agent in field level. Various methods are used to study the effectiveness of biocontrol agents in preventing or controlling the pathogenesis of the pathogen. Seed bacterization of host plant seeds by different biocontrol agents such as *Pseudomonas* and Rhizobacteria have been used (Homma and Suzui 1989; Alström 1991). Seedlings treatment (Wood et al. 1997; Walker et al. 2002), whole plants at green house level (Asaka and Soda 1996; Ghods-Alavi et al. 2012; Siddiqui and Shakeel 2007; des Essarts et al. 2016) and even the tissues are used like leaf assays for pathogens (Dong et al. 2001; Pane et al. 2015; Bais et al. 2004; Walker et al. 2004) tubers assay (Dong et al. 2001; Molina et al. 2003) are few of the biocontrol methods used frequently. Different biocontrol methods popularly used are listed in Table 4 (adapted from Diallo et al. 2011)

Table 4 Diversity of microbial biocontrol agents and mechanism involved in biocontrol

Diseases (Pathogen)	Biocontrol agents	Mechanisms involved	Biocontrol assays
Bacterial wilt/brown rot (*Ralstonia solanacearum*)	*Bacillus polymyxa*	Preemptive colonization	Soil microcosm
	Bacillus subtilis and *Paenibacillus macerans*	Induced systemic resistance	In vitro screening and Soil microcosm
	Fluorescent pseudomonads	Induced systemic resistance, Preemptive colonization	Tuber assay, soil microcosm and field trial
	Nonpathogenic *Ralstonia solanacearum*	Induced systemic resistance	Tuber assay and field trial
Blackleg and soft-rot (*Dickeya* spp./ *Pectobacterium* spp.)	*Bacillus subtilis*	Antibiosis	In vitro screening and tuber assay
	Fluorescent pseudomonads	Antibiosis and iron competition	In vitro screening, tuber assay, soil microcosm, field trial
	Pectobacterium spp.	Competition	Tuber assay
Ring rot (*Clavibacter michiganensis* ssp. *sepedonicus*) Scab (*Streptomyces* spp., mainly *Streptomyces scabiei*)	Fluorescent pseudomonads	Antibiosis, preemptive colonization	In vitro screening and soil microcosm
	Streptomyces bacteriophage	Cell lysis	Tuber assay
	Fluorescent pseudomonads	Not determined	Field trial
	Nonpathogenic Streptomyces	Antibiosis, competition	In vitro screening, tuber assay, soil microcosm and field trial
Fusarium dry rot	*Bacillus* spp.	Antagonism	In vitro screening

			and tuber assay
(*Fusarium* spp., mainly *Fusarium roseum* var. *sambucinum* and some *Fusarium oxysporum*) Late blight/Mildew (*Phytophtora infestans*)	Enterobacter cloacae	Antagonism	Tuber assay and storage
	Fluorescent pseudomonads	Antagonism	Tuber assay and storage
	Hyphal wall components Pseudomonas	Induced systemic resistance	Soil microcosm
	Pseudomonas koreensis or its biosurfactant	Antagonism	Greenhouse trial (leaf assay
	Pseudomonas putida	Antibiosis, competition	Soil microcosm
	Phytophthora cryptogea	Induced systemic resistance	Soil microcosm
Rhizoctonia black scurf and stem canker (*Rhizoctonia solani*)	Binucleate *Rhizoctonia*	Competition	Soil microcosm and field trial
	Rhizoctonia zeae	Competition	Soil microcosm
	Verticillium bigutattum	Mycoparasitism	Soil microcosm and field trial
	Trichoderma harzianum	Competition, induced systemic resistance	Tuber assay, sand and plantlet microcosm
Verticillium wilt (*Verticillium dahliae*)	*Clonostachys rosea*	Mycoparasitism	Soil microcosm
	Pseudomonas fluorescen	Antagonism	Soil microcosm
	Talaromyces flavu	Mycoparasitism	Soil microcosm

1.5 *Bacillus* as quorum quenching based biocontrol agent

The first quorum quenching enzyme encoded by the *aiiA* gene, identified as AHL lactonase was identified from a soil bacterial isolate belonging to a *Bacillus* genus (Dong et al. 2000, 2001). Shortly after that, a range of other bacterial isolates and strains that produce AHL degrading enzymes were identified from soil, plant and biofilm samples as well as from laboratory bacterial culture collections. The pie chart in Figure 6 shows the distribution of quorum quenching enzymes in various bacterial genera. As is evident from the pie chart, maximum reports of quorum quenching enzymes identified, are from *Bacillus* species. Thus, *Bacillus* species are potent biocontrol agents and need to be explored for the same. Although there are numerous reports of quorum quenching enzymes in *Bacillus* sp. (*B. thuringiensis*, *B. cereus*, *B. anthracis*, *B. mycoides*, *B. subtilis*, *B. amyloliquefaciens*, *Geobacillus* sp.) which are mostly characterized for AHL lactonases (Lee et al. 2002; Pan et al. 2008; Yin et al. 2010; Augustine et al. 2010; Seo et al. 2011) only *B. thuringiensis* is explored extensively for its quorum quenching based biocontrol potential (Dong et al. 2001; Dong et al. 2002; Dong et al. 2004). Thus, *Bacillus* genera is a rich pool of bacterial strains producing AHL degrading enzyme and should be further explored as biocontrol agents against quorum sensing pathogens.

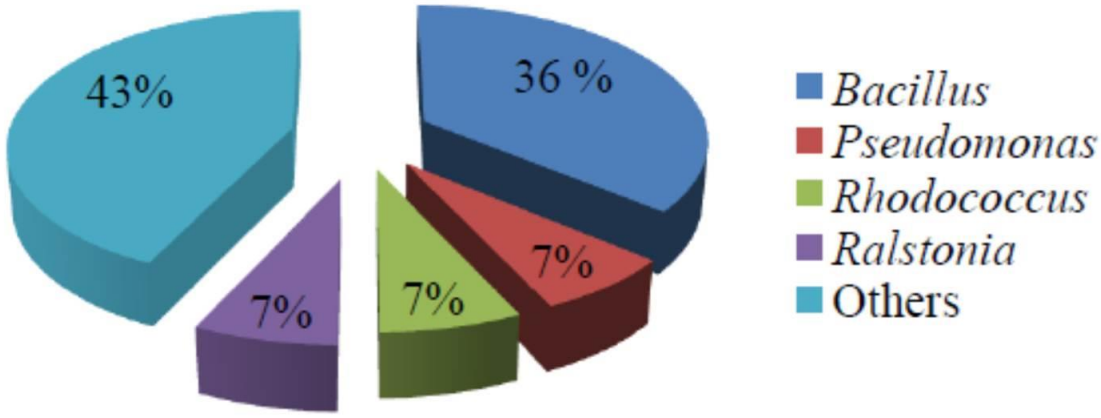

Figure 6 Distribution of quorum quenching enzymes in various bacterial genera

Rationale of the present study

The most commonly exploited biocontrol mechanism against bacterial and fungal plant pathogen is antibiosis through variety of approaches which majorly include competitive root colonization, antibiosis, lytic enzyme production, detoxification and degradation of virulence factors and systemic resistance induction. Usage of any of this mechanism, mainly antibiotics, against pathogen has created a problem of evolving resistance by the pathogens. The finding that quorum sensing could be a widely conserved mechanism in the regulation of virulence suggests that quorum quenching mechanisms might have promising potentials in biocontrol (Dong and Zhang 2005). The quorum quenching based biocontrol approach does not affect the growth of the pathogen rather it inhibits or attenuates the quorum sensing regulated virulence of the pathogen. Thus, this exerts very limited selection pressure on pathogen which excludes the possibility of development of resistance by the pathogen against this approach. Hence, the quorum quenching enzymes could also be explored as a new version of anti-virulence strategy for the biocontrol of microbial infections. *Bacillus* genus is a major source for finding potential isolates which express quorum quenching enzymes. The data suggest that ~ 43% of the quorum quenching bacteria are reported from *Bacillus* genus (Uroz et al. 2009; Dong and Zhang 2005, Czajkowski and Jafra 2009). After the finding of the first quorum quenching enzyme from Gram-positive *Bacillus* species (Dong et al. 2000), a wide range of other bacterial isolates and strains are being screened for the production of AHL degradation enzymes from soil, plant and biofilm samples as well as from laboratory bacterial culture collections.

Taking these aspects into consideration present study involves isolation and screening of *Bacillus* sp. for AHL degradation phenotype and identification of the selected isolates. Moreover, the study also focuses on the properties essential for biocontrol of quorum quenching bacteria such as adherence on seeds, root or plant colonization ability and persistence of these isolates on root (mung bean model), broad host range which is susceptible to *Pcc* (Potato, Carrot and Cucumber) on which the quorum quenching isolates can survive and exhibit the biocontrol potential. Additionally, studies showing absence of deleterious effect of the isolates on the host plant and the ability of quorum quenching isolates to control the disease pre-infection (preventive) and post-infection (curative) are included. The model quorum sensing pathogen used for these studies was *Pectobacterium carotovorum* subsp. *carotovorum* BR1 (Lab isolate obtained in earlier studies in our

laboratory). It is broad host-range pathogen which infects *Daucus carota* (carrot), *Solanum melongena* (brinjal), *Lagenaria siceraria* (bottle gourd), *Cucumis sativus* (cucumber), *Solanum tuberosum* (potato). It produces 3-oxo-C6-HSL as quorum sensing molecule**.** Another quorum sensing pathogen *Pseudomonas aeruginosa* PAO1 was also used as model pathogen. It produces C4-HSL and 3-oxo-C12-HSL and causes infection to *Arabidopsis*, sweet basil and lettuce. Thus, the broad objective of the present study was to identify a broad range quorum quenching *Bacillus* and to evaluate diverse biocontrol properties of selected quorum quenching bacteria for the attenuation of soft rot caused by quorum sensing pathogens. Further interest was to identify the mechanism of AHL degradation exhibited by the selected isolate involved. The studies further expanded to demonstrate the efficacy of a quorum quenching based attenuation of pathogenesis during actual infection. The study of spatial structuring of a quorum quenching isolate and pathogen when grown on plant roots was also conducted as the spatial distribution will determine the importance of where the quorum quenching isolate should be in order to break down pathogen produced AHLs. The distance between the AHL degrading strain and pathogen is likely to be a crucial determinant in deciding whether the virulence is attenuated and if therefore the quorum quenching is effective.

In this background the objectives framed for present study were as follows,

1. **Isolation of special strains of Bacillus spp. possessing AHL degrading activity and their identification**

2. **Characterization of the selected AHL degrading Bacillus isolates for biocontrol of soft rot caused by Pectobacterium carotovorum subsp. carotovorum**

3. **Characterization of the quorum quenching mechanism of the selected isolate and efficacy of quorum quenching as biocontrol approach**