Chapter 1

Literature Review and Introduction

1.1 Biocontrol as a strategy against phytopathogens

Crop pests (diseases, insects, and weeds) cause projected losses of 40% to yearly global crop despite the use of approximately 3 billion tons of chemical pesticides worldwide (Messing & Brodeur, 2018). Chemical pesticides have definitely added to improved crop yield since the mid-1900s, but abuse and dependency on pesticides has led to environmental worries and an occurrence of pesticide-resistant pests. The invention and commercialization of new synthetic pesticides is progressively more challenging and pricier; over 140,000 compounds might be investigated to create one new commercially suitable pesticide after 10 years of work and more than US $250 million (Glare et al., 2012). Therefore, the advancement of substitute pest control measures has become a significant for maintainable crop production and decrease of pesticide use to a bare minimum (Marian & Shimizu, 2019).

To reduce dependency on chemical control Integrated pest management (IPM) is now acknowledged method. An important aspect of IPM is biocontrol using beneficial microorganisms. Growing interest in the utilization of microbial biocontrol agents (MBCAs) to regulate plant pathogens is demonstrated by the vast literature available (Marian & Shimizu, 2019). Quorum Quenching is a part of IPM. Furthermore, many big international companies have established a strong interest in making microbial biocontrol products (MBPs) by obtaining small biopesticide companies and signing licensing agreements to allocate and sell MBPs made by smaller companies (Pelaez et al., 2017). In 2012, the global biopesticide market was rising at a 15.6% annual growth rate (Glare et al., 2012); 10 MBCAs were listed between 1996 and 2000 in the European Union (EU) (Droby et al., 2016), and 27 microbial fungicide products were accepted in the last 15 years (Marian & Shimizu, 2019) which is an encouraging sign towards MBP usage.

1.2 An overview of Quorum Sensing

Quorum sensing (QS) is a cell population density-based mechanism which controls the gene expression in bacteria related to wide-ranging functions like virulence, bioluminescence, biofilm formation, competence development etc. in response to a threshold level concentration of a diffusible signaling molecule produced by the bacteria on accumulation of sizable population. Gram-positive and gram-negative

bacteria both exhibit quorum sensing but the QS mechanisms are unlike each other and are contrasting in nature (Miller & Bassler, 2001; Rutherford et al., 2012; Helman et al., 2014; Baltenneck et al., 2021). Gram-negative bacteria regulate their quorum sensing pathways by a small autoinducer molecule called N-acyl Homoserine Lactone (AHL) signaling molecule while gram positive bacteria use autoinducing oligopeptides (AIPs) signaling molecules to regulate their quorum sensing mechanisms (Fig. 1.1) (Waters & Bassler, 2005; Rutherford et al., 2012; Zhou et al., 2017). Gram-negative bacteria and their quorum sensing systems have been widely studied in the last couple of decades. *Vibrio fischeri, Vibrio harveyi, Pectobacterium carotovorum* (previously *Erwinia carotovora*), *Pseudomonas* sp., *Agrobacterium tumefaciens, Burkholderia* sp., *Chromobacterium violaceum, Ralstonia solanacearum, Rhizobium leguminosarum, Salmonella typhimurium, Serratia liquefaciens, Yersinia pestis* etc. are few gram-negative bacteria which use quorum sensing as a mechanism for their various functions (Miller & Bassler, 2001; Boyer et al., 2009) out of which *Pectobacterium* sp., *Pseudomonas* sp., *Ralstonia solanacearum* (uses (R)-methyl 3-hydroxymyristate (3-OH MAME) as a QS signal, and not AHL), *Agrobacterium tumefaciens, Pantoea stewartii, Xanthomonas campestris* pv. *campestris* are well reported phytopathogens which use the QS mechanism to cause virulence against plants (von Bodman et al., 2003). Such bacteria generate and release Plant Cell Wall Degrading Enzymes (PCWDEs) such as pectate lyase, pectin lyase, polygalacturonase, protease and cellulase that are QS regulated, causing diseases in plants(Põllumaa et al., 2012; Moleleki et al., 2017).

Any method that disrupts the quorum sensing signaling, thwarting the gene expression leading to loss of its function is termed as Quorum Quenching (QQ) (Grandclément et al., 2016). On a broader term it is also known as Quorum Sensing Inhibition (QSI) as there are various mechanisms by which the process of quorum sensing in bacteria can be repressed (Kalia, 2013). Quorum quenching approaches and mechanisms against AIPs produced by gram-positive bacteria have been studied extensively by (Singh et al., 2016). QQ in Gram-negative bacteria can be brought about by chemicals (Borges et al., 2014), external changes (pH or temperature) (Papenfort & Bassler, 2016) or enzymes (Kalia, 2013; Grandclément et al., 2016). The enzymatic quorum quenching activity against gram-negative bacteria has been reported by various genera including those belonging to Actinomycetota. The quorum quenching enzymes are of three

major types i) AHL lactonases ii) AHL acylases and iii) AHL oxido-reductases. They act on the signal molecule AHL, produced by quorum sensing gram-negative phytopathogenic bacteria and deactivate it, which in turn prevents the production of Plant Cell Wall Degrading Enzymes (PCWDEs) which are the chief virulence factors of the pathogen that cause the damage to the plants resulting in soft rot (Kalia & Purohit, 2011; Uroz et al., 2014; Garge & Nerurkar, 2016; Polkade et al., 2016). Other than enzymatic quorum quenching, quorum sensing inhibitors are also present. QQ also occurs via chemical Extensive reviews on the topic of quorum quenching mechanism can be found in literature (Zhang et al., 2004; Kalia & Purohit, 2011; Uroz et al., 2014).

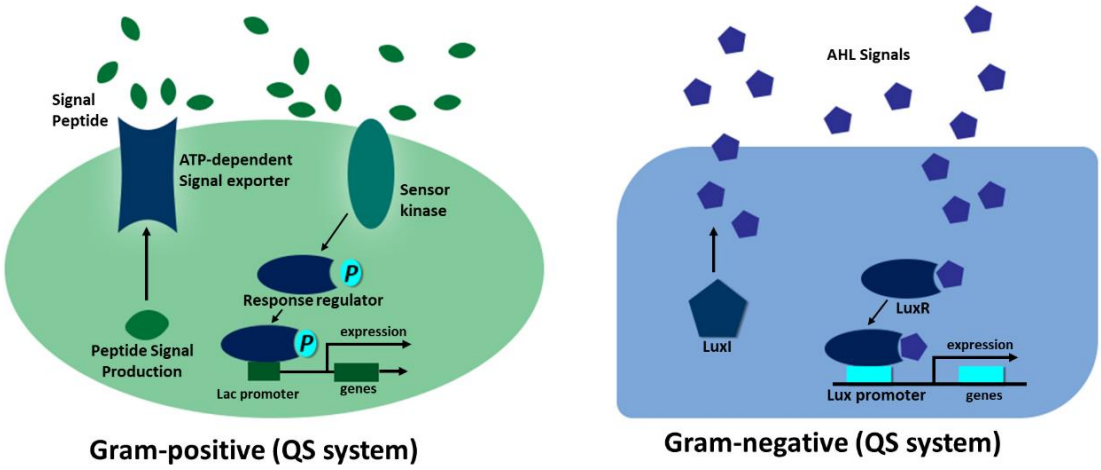

Figure 1.1 Quorum sensing systems in bacteria

1.3 Quorum Sensing in Pathogens

Quorum sensing was first reported in bacteria *Vibrio fischeri* and *Vibrio harveyi* around 45 years ago (Nealson et al., 1970), though the precise words were not used at that time. Later on, transcriptional regulator LuxR and the AHL synthase LuxI were established quorum sensing regulators for bioluminescence in *V. fischeri* and *V. harveyi* in a cell density dependent manner. The signaling molecule AHL is synthesized by the *lux*I gene product, autoinducer synthase. *lux* R and *lux*I mediate the cell density dependent control of transcription of the genes in *lux* operon (Fuqua et al., 1994). The primary model involves the stimulation of gene expression by LuxR protein when it attaches to their promoter after it is combined with AHL (Fig. 1.2) (Loh et al., 2002).

AHL molecules generated by different bacteria are of not of same lengths depending on number of carbons in them and the presence and absence of oxygen atom in the structure (Ng & Bassler, 2009). Gram-negative phytopathogenic bacteria mostly have AHL based quorum sensing systems which regulate the virulence in plants with the exception of *Xanthomonas campestris* which has a DSF based quorum sensing system (Cai et al., 2017). The common gram-negative phytopathogenic bacteria that possess quorum sensing regulated virulence mechanism are listed in Table 1.1. On the other hand, the Gram-positive bacterial pathogens such as *Staphylococcus aureus, Clostridium botulinum, Enterococccus faecalis, Bacillus subtilis, Lactobacillus plantarum, Streptococcus* sp. use AIPs as signaling molecules (Teixeira et al., 2013; O'Rourke et al., 2014; Rizzello et al., 2014; Ihekwaba et al., 2015; Wolf et al., 2016; Ishii et al., 2017).

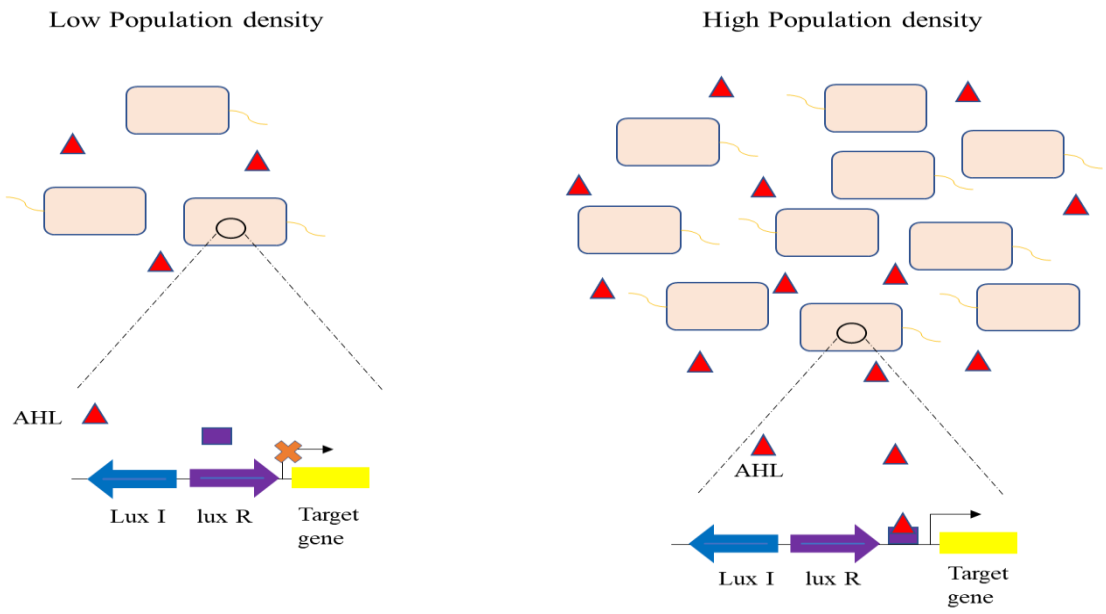

Figure 1.2 Quorum sensing mechanism in Gram-negative bacteria

Table 1. 1 Phytopathogenic quorum sensing bacteria

Organism	Regulatory proteins	Major signal molecule	Virulence factors	References
Agrobacterium tumefaciens	TraI/TraR	3-Oxo-C8-AHL	Ti plasmid conjugation	Lang & Faure, 2014
Burkholderia glumae	EanI/EanR	C8-AHL	Production of EPS and infection of onion leaves	Chen et al., 2015; Gao et al., 2015; Jang et al., 2014

Dickeya dadantii	VfmE/VfmI	3-Oxo-C6-AHL	Plant cell wall degradation	Nasser et al., 2013
Pantoea stewartii	EsaI/EsaR	3-Oxo-C6-AHL	Production of EPS and Pectate lyases	Burke et al., 2015
Pectobacterium carotovorum subsp. *carotovorum*	ExpI/ExpR CarI/CarR	3-Oxo-C6-AHL	Virulent Exoenzymes	Ham, 2013; Joshi, et al., 2016
Pectobacterium carotovorum subsp. *atrosepticum*	ExpI/VirR	3-Oxo-C6-AHL	Production of virulent extracellular enzymes	Monson et al., 2013
Pseudomonas syringae	AhlI/AhlR	3-Oxo-C6-AHL	Production of EPS	Scott & Lindow, 2016; Yu et al., 2014
Ralstonia solanacearum	SolI/SolR PhcB/RalA	C8-AHL	Action of ralfuranone, resulting in plant virulence	Mori et al., 2018
Xanthomonas campestris	RpfC/RpfG	DSF	Plant virulence	Cai et al., 2017

1.4 Quorum sensing regulation of virulence factors in *Pectobacterium* species

Pectobacterium sp. are plant pathogens having a diverse host range, comprising agriculturally important crops like carrot, potato, cucumber, eggplant (brinjal), tomato, leafy greens etc. *P. carotovorum* and *P. atrosepticum* have been listed as one of the top ten phytopathogens (Mansfield et al., 2012). *Pectobacterium carotovorum* subsp. *carotovorum* and other *Pectobacterium* spp. such *as P. wasabiae, P. carotovorum* subsp. *atrosepticum* cause two diseases: soft rot disease as an outcome of damage to the succulent parts of the plant like fruits, stem, tuber, bulbs etc. leading to necrosis and Blackleg which is caused by the rotting mother tuber or seed that spreads to the stem and other parts of the plant. The phytopathogen relies upon production of PCWDEs, like cellulases, hemicellulases, proteases and pectinases which hydrolyze the cellulose and pectin that are components of plant cell wall that keep the plant together. The PCWDEs disrupt the plant cell integrity that ultimately breaks up the plant structure and leads to the destruction of the tissue. Soft rot starts as fluid-soaked lesions on plant parts that rise in size rapidly. The plant tissue softens and collapses causing fluid to ooze out keeping the skin intact sometimes. The decaying tissue is

creamy, blackish or brownish and emanates foul smell (Fig. 1.3(A, B, E) and 1.4). Depending upon the vulnerability of the host and potency of the bacterial strain, different levels of rotting is detected in the host; though factors such as temperature and humidity also play a significant role in the activity of rotting.

Blackleg is caused by both *Pectobacterium* and *Dickeya* species is a slimy, wet, black rot lesion spreading from the rotting mother tuber up the stems, especially under wet conditions. However, when conditions are dry, symptoms tend to be stunting, yellowing, wilting and desiccation of stems and leaves. Late in summer, under persistent rainy conditions, extensive stem rot, starting from the top and further progressing downwards to the base, can develop and can be confused with the symptoms of blackleg, but is usually caused only by *Pcc* (Fig. 1.3(C, D) and 1.5) (Czajkowski et al., 2011b; Ham, 2013; Monson et al., 2013; Põllumaa et al., 2012).

Soft-rot bacteria survive in infected fleshy organs in storage and in the field, in debris, on roots or other parts of host plants, in ponds and streams used for water irrigation, seldom in the soil, and in the pupae of many insects (Fig. 1.6). The disease may first arise in the field on plants grown from formerly infected seed pieces. Some tubers, rhizomes, and bulbs become infected via wounds or lenticels after they are created in the soil. The inoculation of bacteria into fleshy organs and their additional diffusion in storage and in the field are enabled greatly by insects.

Soft-rot bacteria can live in all stages of the insect. Moreover, the bodies of the insect larvae (maggots) become diseased with bacteria when they are crawling about on rotting seeds, carry them to healthy plants, and put them into wounds where they can initiate the disease. Even when the plants or storage organs are unaffected to soft rot and can stop its development by forming woundcork layers, the maggots damage the wound cork as fast as it is formed and the soft rot ensues. When soft-rot bacteria enter wounds, they feed and increase at first on the liquids released by the broken cells on the wound surface. There they produce large amounts of pectolytic enzymes that break down the pectic elements of the middle lamella and bring about maceration of the tissues. Because of the high osmotic pressure of the macerated tissue, water from the cells diffuses into the intercellular spaces; as a result, the cells plasmolyze, break down, and die. Bacteria keep on moving and multiply in the intercellular spaces, while their enzymes go ahead of them and prepare the tissues for attack. The invaded tissues become soft and are converted into a slimy mass containing of countless bacteria

swimming about in the liquefied materials. The epidermis of most tissues is not attacked by the bacteria; however, cracks are usually present, and the slimy mass extrudes through them into the soil or in storage, where it interacts with other fleshy organs, which are then infected (Agrios, 2005).

Figure 1.3 Bacterial soft rots of fruits and vegetables caused primarily by species of *Pectobacterium*.

Where (A) Lenticel infection of potato tuber leading to soft rot. (B) Stem-end rot of potato tubers induced by *Pectobacterium carotovorum* subsp. *atrosepticum*, the cause of potato blackleg. (C) Potato plant infected with blackleg. (D) Potato plants in the field showing blackleg symptoms. (E) A new potato showing bacterial soft rot at harvest time. (Agrios, 2005) Permission taken from Elsevier to use this image, License Number 5226271244881.

Figure 1.4 Bacterial soft rot of vegetables on (A) cabbage head, (B) carrots, (C) tomato, (D) onion bulb, and (E) celery.

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Figure 1.5 Bacterial wilt and stem rot of fleshy plants caused by *Dickeya chrysanthemi* and *Pectobacterium* sp. respectively (A) Stem rot of corn. (B) Canker and stem rot of cassava and (C) stem rot of cassava. (D) *Pectobacterium* stem rot of banana in close-up showing internal stem discoloration (E) and in the field.

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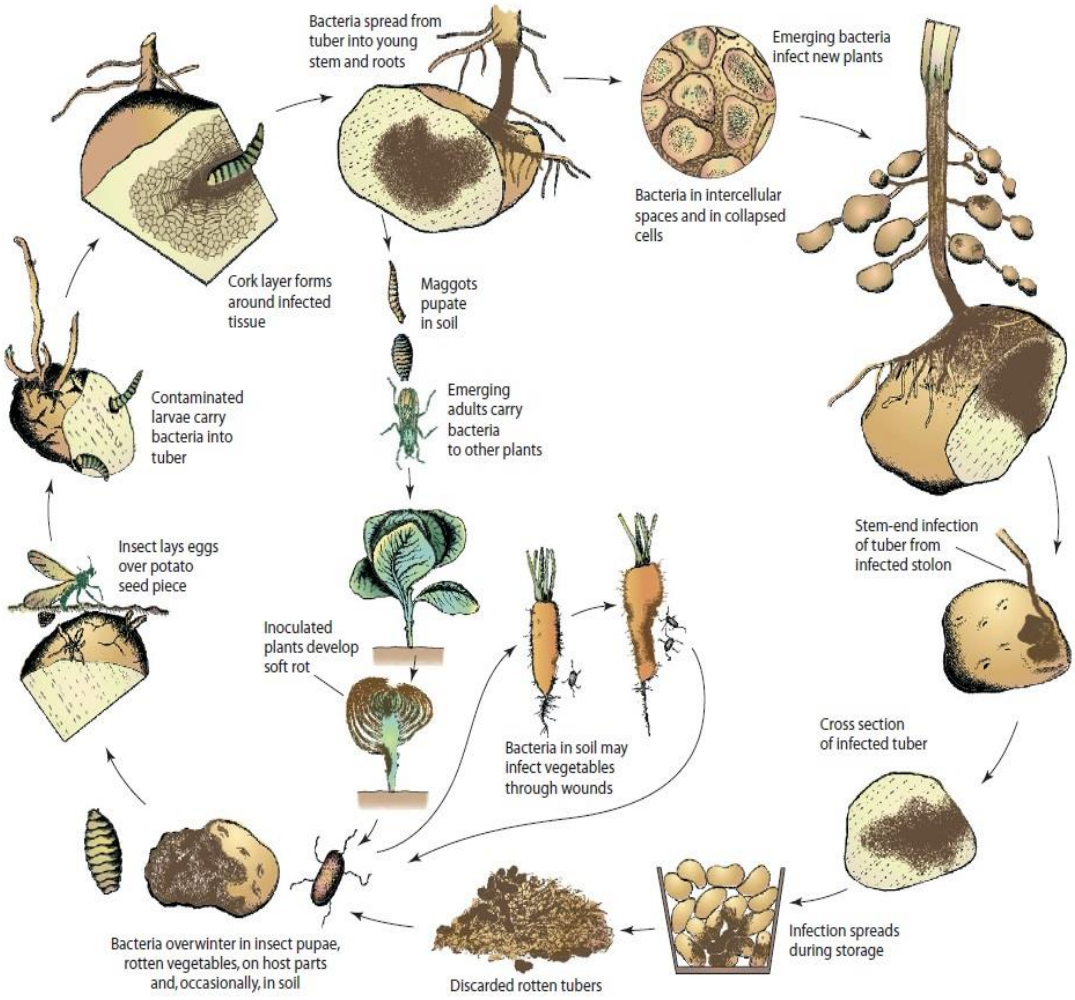

Figure 1.6 Disease cycle of bacterial soft rot of vegetables caused by soft-rotting *Pectobacterium* sp.

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The soft rot causing *Pectobacteria* have been divided into two classes on the basis of AHL they produce: Class I strains produce N-3-oxooctanoyl-L-homoserine lactone (3-O-C8-AHL) and low quantity of 3-oxohexanoyl-L-homoserine lactone (3-O-C6-AHL). On the other hand, Class II strains mainly produce 3-O-C6-AHL, whereas little or none of 3-O-C8-AHL (Rajesh & Rai, 2016). *Pectobacteria* have a complex regulation of its quorum sensing system. Quorum sensing controls the regulation of plant cell wall degrading enzymes (PCWDEs), Type three secretion system (T3SS) and Carbapenem antibiotic production in *Pectobacteria*. ExpI, is the LuxI synthase homolog in *Pectobacteria* and can synthesize 3-O-C6-AHL or 3-O-C8-AHL CarR, ExpR1 and ExpR2 are LuxR homologs in *Pectobacteria*. ExpI interacts with CarR, ExpR1 and ExpR2 once the AHLs mount up and achieve a threshold concentration

limit (Joshi et al., 2016).The synchronized production of the virulence factors of *Pectobacteria* is through two main pathways i) the AHL system and ii) GacS/Rsm system. As shown in Fig. 1.7, two LuxR homologs, ExpR1 and ExpR2, upregulate RsmA at low levels of AHLs and directly inhibit PCWDEs production and the AHL synthase ExpI. rsmA is the global repressor gene that regulates extracellular enzymes and pathogenicity in soft rot causing *Pectobacteria* (Cui et al., 1995). RsmA belongs to a post transcriptional Rsm system and is responsible for the destabilization of mRNA transcripts that encode PCWDEs. GacA/GacS system is active at high cell density and promotes the transcription of rsmB, a non-coding RNA which is also part of Rsm system and blocks the RsmA activity by sequestration, allowing translation of rsmA-targeted mRNAs. At adequate levels, 3-O-C8-AHL bind to ExpR1, whereas 3-O-C6-AHL binds to ExpR2. When AHLs bind to ExpR1 and ExpR2, they inhibit the expression of rsmA, as a result, the mRNA transcripts that encode PCWDEs become free and the enzymes are expressed. A two-component system of ExpS (sensor kinase) and ExpA (response regulator) regulates the transcription of rsmB (Valente et al., 2017). ExpS and ExpA are homologs of GacS and GacA of various other Gram-negative bacteria. In *Pectobacterium* sp. quorum sensing synchronizes the virulence for prolific infection through synergistic negative regulation of ExpR1 and ExpR2. On the other hand, the communication of AHL with CarR regulator is much simpler. CarR binds to the 3-O-C6-AHL and then binds the carA promotor, which controls the Car operon that encodes the Carbapenem antibiotic. (Mole et al., 2007; Tichy et al., 2014). The quorum sensing system gets more complicated due to the expression of many PCWDEs which are regulated positively by the breakdown of products of the pant cell wall produced by the activity of the bacterial pectinases on the tissues of plants (Fig. 1.7). These products include 5-keto-4-deoxouronate, 2,5-diketo-3-deoxygluconate and 2-keto-3-deoxygluconate (KDG). In the presence of these metabolites, regulation occurs due to the discharge of the transcriptional repressor KdgR. Operators of many of the PCWDEs and rsmB genes contain the binding sites for KdgR repressor. As a result, the initial production of pectinases causes further initiation of virulence genes both transcriptionally as well as post-transcriptionally (Valente & Xavier, 2016). Breakdown products of the substrates released by the action of the bacterial PCWDEs also perform the role of signaling molecules for the plant, indicating the presence of a pathogen that prompts the hypersensitive disease response. Quorum sensing places

pathogenicity associated genes under density dependent control which avoid the activation of host plant's defense systems (Newton & Fray, 2004). A relatively high inoculum of *Pectobacterium* sp. is required for successful infection, and the progression of the disease is then a race between the development of plant resistance and bacterial multiplication. Thus, the production of PCWDEs prematurely when the cell densities are low gives increase to an unsuccessful infection and on the contrary would induce local and systemic plant defense response, which in turn would resist successive infections. Cleverly, the *Pectobacterium* sp. uses AHLs to measure its cell density and starts a pathogenic attack only when its population density is above a critical level, which makes sure that there is a high probability of preventing host resistance (Põllumaa et al., 2012). Thus, it offers the pathogen a finely adjusted temporally controlled approach to overpower the host by escaping the host response as well as profusely producing the virulence factors.

In experiments with *Pectobacterium carotovorum* mutant, it was observed that inactivation of ExpI resulted in less production of PCWDEs, inability to produce AHL and overall reduction in virulence in potato tubers and stems (Moleleki et al., 2017). Gene expression analysis proved that flagella are also a part of the QS regulon and is positively regulated while fimbriae and pili are negatively regulated by QS. Various pathogenicity connected factors (genes) which work within the quorum sensing system are necessary for the virulence of *Pcc*, these factors were examined and grouped into nutrient consumption (pyrD, purH, purD, leuA and serB), production of plant cell wall degrading enzymes (PCWDEs), (expI, expR) motility (flgA, fliA and flhB), biofilm formation (expI, expR and qseC) and vulnerability to antibacterial plant chemicals (tolC) (Lee et al., 2013). Bearing in mind the important role QS plays in *Pcc* virulence, a diversity study was initiated in which strains were isolated from soil and characterized by species specific PCR and 16S rRNA sequencing, pathogenicity on different hosts, virulence enzyme production and biochemical characteristics; out of these a highly virulent *Pectobacterium carotovorum* subsp. *carotovorum* BR1 strain showing broad host specificity was obtained (Maisuria & Nerurkar, 2013). This strain was later used for QQ studies throughout this research work. Virulence determinant enzymes polygalacturonase (Maisuria et al., 2010) and pectate lyase (Maisuria & Nerurkar, 2012) of *Pcc*BR1 were purified and biochemical characterization as well as thermodynamic characterization was carried out.

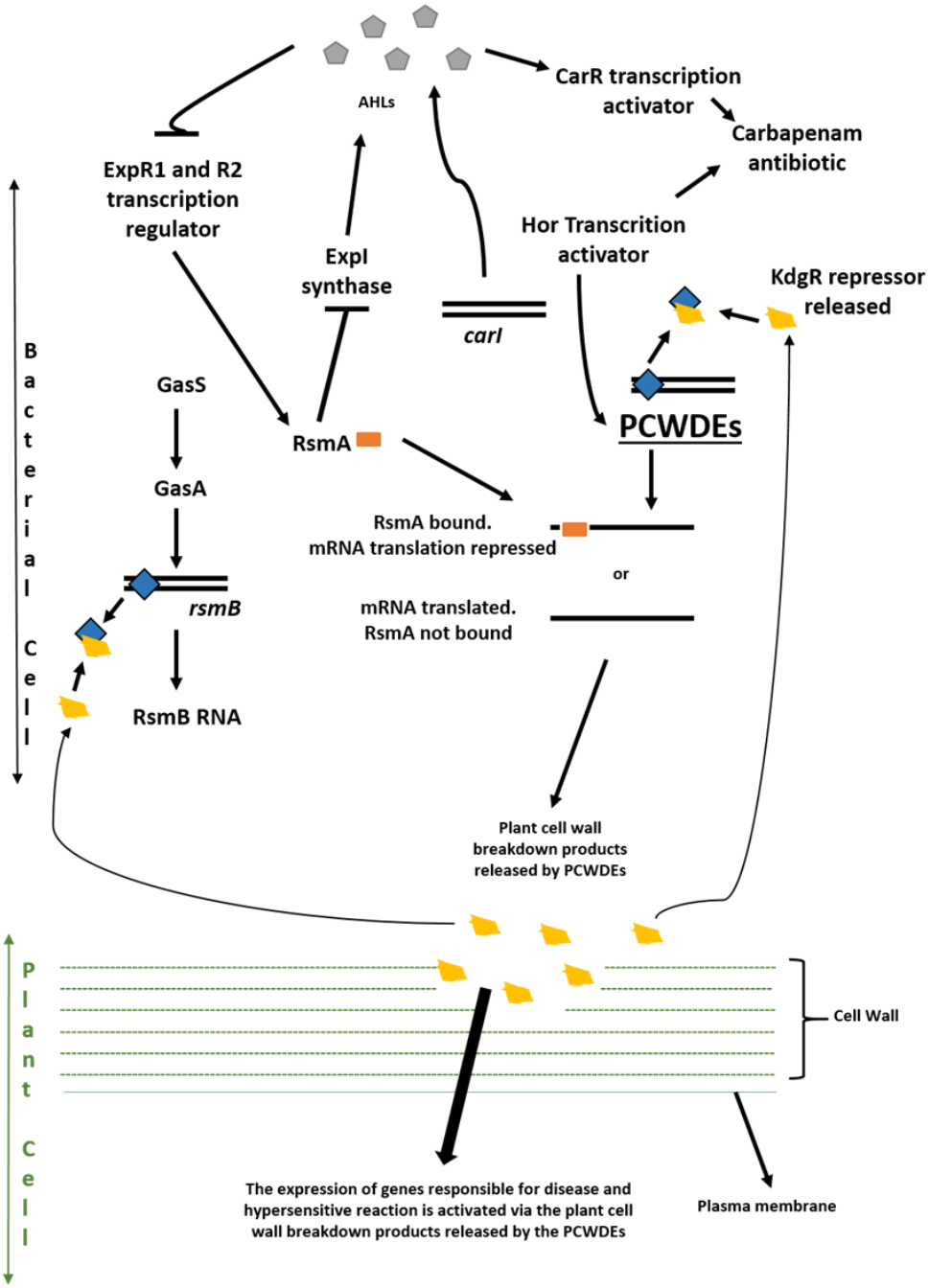

Figure 1.7 Common regulatory systems controlling virulence in *Pectobacterium carotovorum* subsp. *carotovorum*.

In *Pectobacterium atrosepticum*, VirR is the LuxR-type repressor which controls virulence of the bacteria. Here, VirR is auto-repressing which in turn triggers the transcription of rsmA in the absence of AHL. VirR also controls the production of siderophores and swimming motility. Thus, VirR, a LuxR-type protein operated as activator and repressor of transcription in vivo without the signalling molecule AHL

(Monson et al., 2013). Again, in *Pectobacterium atrosepticum,* it was found that by moderating RsmA levels, (p)ppGpp applied regulation through the control of the RsmA antagonist, RsmB and thereby exert a QS controlled virulence in the bacteria. It was also observed that the ratio of RsmA protein to its RNA rival, rsmB, was controlled independently by QS and (p)ppGpp. Surface swarming motility of *Pectobacterium atrosepticum* was also controlled by QS. It required motility and O antigen biosynthesis for the process (Bowden et al., 2013).

1.5 Bacterial biosensors for studying quorum sensing

Different kinds of AHL molecules produced by bacteria can be marked due to advancement of bioassays based on lux or LacZ reporter constructs which has also helped in identification of LuxI homologues in many bacteria responsible for AHL synthesis (Swift et al., 1993). Bacterial biosensors can detect exogenously supplied AHL which helps in positive regulation of reporter gene for example Bioluminescence, Green fluorescent protein, Violacein pigment production etc. Such biosensor strains lack the ability to produce AHL but contain functional LuxR family protein which can activate transcription of reporter gene after binding with exogenously provided AHL. LuxR family protein has specificity towards cognate AHL molecules while some LuxR family proteins can identify AHLs having acyl chains of C4 to C8 in length. McClean et al., (1997) constructed a mutant *Chromobacterium violaceum* CV026 strain which acts as biosensor to detect external AHL production. Wild type *Chromobacterium violaceum* has QS system which controls production of purple coloured pigment violacein via CviI/R AHL system. *C. violaceum* CV026 cannot produce its own AHL due to insertion of miniTn5 transposon in cviI AHL synthase gene while another transposon is also inserted in violacein repressor locus. CviR AHL binding transcription activator is functional hence after exposure of *C. violaceum* CV026 strain to exogenous AHL, visually clear purple pigmentation is seen. *C. violaceum* CV026 strain shows specificity towards C6 AHLs whereas shorter or longer acyl chain AHLs like C3/C4 or C10 AHLs have little or no effect on pigment production (Fig. 1.8A). Various other biosensors use plasmid constructs which harbors LuxCDABE operon of *Photorhabdus luminiscens* producing bioluminescence as response in reporter system (Fig. 1.8B) (Winson et al., 1998). Such plasmids are transformed into *E. coli* cells which do not produce AHL to detect exogenous AHL.

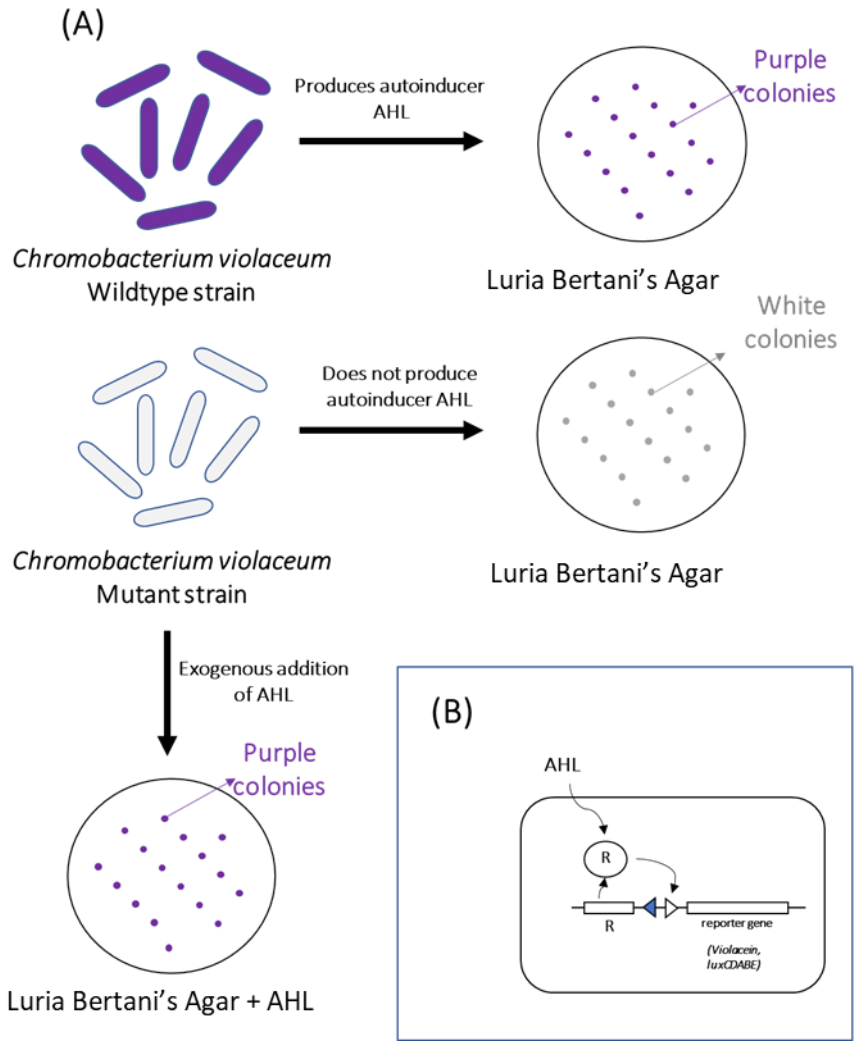

Figure 1.8 (A) CV026 application as a biosensor, (B) Response of external AHL by CV026

1.6 Quorum quenching against phytopathogenic bacteria

Having discussed the tactical position of the quorum sensing systems in regulation of virulence, quorum sensing blockage can be an efficient approach to attenuate the virulence of these phytopathogens. This has been confirmed in transgenic plants that displayed resistance to infection by *Pectobacterium carotovorum* subsp. *carotovorum* when the AHL degrading enzyme gene was cloned (Helman et al., 2014). Any procedure that inhibits or blocks the process of quorum sensing in any manner is known as Quorum Quenching. Quorum quenching is an efficient mechanism to keep in check pathogens (usually phytopathogens) that control their virulence by quorum sensing. In the current scenario of evolving resistance of pathogens to antibiotics and

drugs has required us to look into alternate strategies and by quorum quenching based biocontrol approach the pathogenicity of the bacteria can be controlled without killing the bacteria or the bacteria developing resistance to the biocontrol agent. By not affecting the growth of the pathogen but by inhibiting the quorum sensing regulated virulence of the pathogen, this strategy applies very limited selection pressure on the pathogen (Zhang et al., 2004; Vesuna & Nerurkar, 2018). Cui et al., (2005) have discussed the targets in quorum sensing circuit that can be interrupted to inhibit the pathogenesis of bacterial plant pathogens as depicted in Fig. 1.9. They are categorized as i) QS signal (AHL) inactivation by degrading enzymes (Chen et al., 2013). ii) AHL-LuxR binding inhibition with AHL analogs as antagonists (Koch et al., 2005). iii) Interruption of the AHL biosynthesis pathway either inhibiting the specific AHL synthase or other enzymes in the pathway of AHL biosynthesis precursors (Uroz et al., 2008). Thus, quorum quenching against signal molecule induced quorum sensing phytopathogens have been derived from bacteria as well as eukaryotes (Kalia, 2013). Based on their mechanisms they fall into two major categories as follows.

1) Chemical compound which prevents the function of the quorum sensing system. This type of quorum quenching is usually termed as chemical interference-based quorum sensing inhibition and such chemical compounds are termed as Quorum Sensing Inhibitors (QSI). These can be inhibitors of the targets mentioned in ii) and iii) above.

2) The more frequently studied and explored type of quorum quenching is enzyme based which depends directly on disrupting the quorum sensing signal molecule AHL by different bacterial enzymes.

This study focusses on enzymatic quorum quenching as well as quorum sensing inhibition by phytochemicals, its mechanisms and the work done in this field in detail in relation to phytopathogenic bacteria *Pcc*BR1.

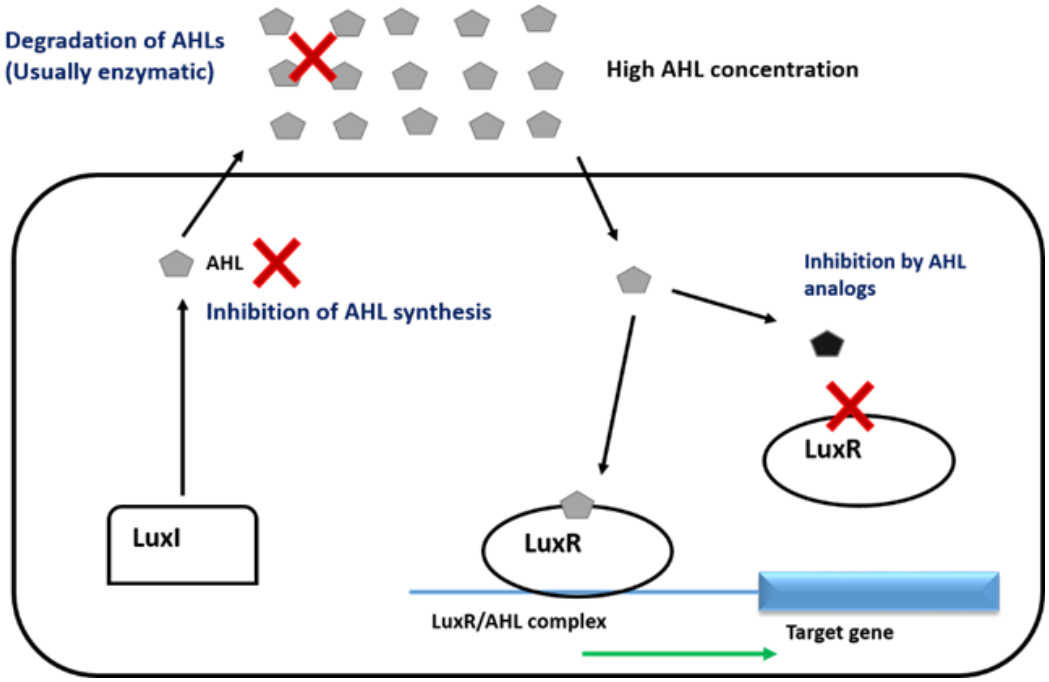

Figure 1.9 Interruption of quorum sensing circuit at different targets to inhibit the virulence gene expression in phytopathogenic bacteria.

1.6.1 Chemical interference-based quorum quenching

Chemical compounds from natural products have been known to act as QSIs. (Defoirdt et al., 2013) have done a detailed review on all different chemical compound based QSIs. Some examples of such compounds which prevent the quorum sensing induced virulence of phytopathogenic bacteria are discussed here. A study revealed that extracts from the plant *Centella asiatica* were useful QSIs against *P. aeruginosa*. It was found out that the *C. asiatica* extracts had ample number of flavonoids in them and they had shown specificity towards the las and rhl QS systems in *P. aeruginosa* (Vasavi et al., 2016). In another study, various plants extracts were screened and identified for their QSI activity against *P. aeruginosa* and it was found out that three compounds; trans-cinnamaldehyde, tannic acid and salicylic acid were found to be potential QS inhibiting compounds. Trans- cinnamaldehyde was found out to be most efficient of the three as per further studies (Chang et al., 2014). ZnO nanoparticles have been reported to reduce elastase, pyocyanin and biofilm formation, molecules and process that are regulated by QS in *P. aeruginosa* strains, in turn reducing the QS based virulence in the bacteria and was suggested as an alternative treatment against *P. aeruginosa* (García-Lara et al., 2015).

Some volatile organic compounds (VOCs) produced by bacteria have also been known to been used as QSI. (Helman et al., 2014), in their review stated and reported a group of volatile suphur compounds like dimethyl sulphide, dimitheyl disulphide(DMDS) and dimethyl trisuphide, produced by bacteria subduing growth of *Agrobacterium* strains. Dimitheyl disulphide also reduced the amount of AHLs produced by various *Pseudomonas* sp. lowering their virulence and pathogenicity.

Most reports of QSIs produced either by plants or bacteria have been reported against phytopathogenic AHL signal molecule-based quorum sensing system of different *Pseudomonas* sp. Supporting this claim, a study revealed that the use of cranberry extract rich of proanthocyanidins (cerPAC) was a very efficient anti-virulence agent against *P. aeruginosa* as it interfered with its QS system. The AHL synthases LasI/RhlI and LasR/RhlR, the QS transcriptional regulators were suppressed and antagonized by the cerPAC (Maisuria et al., 2016). The phytopathogenic bacterium *Pseudomonas savastanoi'*s QS system was inhibited by polyphenolic extracts from plants such as *Olea europaea, Cynara scolymus and Vitis vinifera* without hampering the viability of the bacteria (Biancalani et al., 2016).

Xanthomonas oryzae pv. *oryzae* a causative agent of bacterial rice leaf blight, uses DSF signal molecule-based quorum sensing system for its virulence. By inhibiting the QS pathway and the histidine pathway which is involved in QS circuit in *X. oryzae*, bismerthiazol, a thiadiazole molecule reduced the rice leaf blight disease (Liang et al., 2018).

Phytophenolic compounds have been widely shown to act as quorum sensing inhibitors (QSI) in potential biocontrol strategy against pathogens. Polyphenols are secondary metabolites universally dispersed in all higher plants, which play important roles to protect against plant pathogens. Key compounds that are accountable for antimicrobial activity from plants include phenolics, phenolic acids, quinones, tannins, coumarins, terpenoids, and alkaloids. The first identified QQ compounds were halogenated furanones from benthic marine macroalga *Delisea pulchra*. They bound competitively to the LuxR type proteins to inhibit the QS-regulated behaviours. Thus, promoting their rate of proteolytic degradation without harming the bacteria for their role in inhibiting biofilm formation. Anti-biofilm activity toward various pathogens was also observed in plant constituents like naringenin, salicylic acid, ursolic acid, cinnamaldehyde, methyl eugenol, extracts of garlic and edible fruits. A substantial

decrease in QS-dependent phenotypes such as violacein production, biofilm formation, exopolysaccharide (EPS) production, motility, and alginate production in a concentration-dependent way was done by Quercetin. It acted as a competitive inhibitor for signaling compound to lasR receptor pathway. Additionally, Quercetin notably inhibited biofilm formation and production of virulence factors, including pyocyanin, protease, and elastase at a lower concentration. Carvacrol, one of the main antimicrobial consituents of oregano oil, prevented the formation of biofilms of *C. violaceum*, *Salmonella enterica* subsp. *Typhimurium* and *Staphylococcus aureus*. Moreover, it decreased expression of civil (a gene coding for the N-acyl-L-homoserine lactone synthase), production of violacein, and chitinase activity (both regulated by QS). Clove oil had QQ therapeutic function against *P. aeruginosa* PAO1 and *A. hydrophila* WAF-38. Sub-inhibitory concentrations of the clove oil showed substantial decrease of las-regulated and rhl-regulated virulence factors: LasB, total protease, chitinase, and pyocyanin production, swimming motility, and EPS production (Asfour, 2018). Volatile essential oils, carvacrol and eugenol, interefered with QS, the master regulator of virulence in *Pectobacterium aroidearum* PC1 and *Pectobacterium carotovorum* subsp. *brasiliense* Pcb1692, causing in effective inhibition of QS genes, biofilm formation and PCWDEs, thus resulting in weakened infection (Joshi et al, 2016). Salicylic acid treatment was used to prevent blackleg caused by *Dickeya solani* in potato plants (Czajkowski et al., 2015b). Many such plant compounds have been reported to inhibit QS based virulence in plant pathogens.

1.6.2 Enzymatic quorum quenching for virulence attenuation

The process of quorum sensing and how the mechanism is used by different Gram-negative phytopathogenic bacteria for virulence function is discussed in Section 1.3 and 1.4. Quorum quenching bacteria produce enzymes that interfere with the AHL based quorum sensing in Gram-negative phytopathogenic bacteria as mentioned there. A wide diversity is observed amongst quorum quenching bacteria even though nearly 50% are covered by *Bacillus* spp. An extensive account of the diversity of QQ bacteria is presented by (Chen et al., 2013) where they have given the phylogenetic relationship of the QQ enzymes and have noted that quorum quenching enzymes are present in both the QS and non-QS bacteria. *Bacillus* species are usually not harmful when added as biocontrol agents to the soil. *Bacillus* sp. has been given the designation of GRAS

(Generally Regarded as Safe) by the FDA which affords an added advantage of using them to control QS mediated virulence and hence their QQ ability has been studied in great detail. (Koul & Kalia, 2017).

Enzymatic quorum quenching depends upon bacteria producing enzymes which attack the signaling molecule N-acyl Homoserine lactone (AHL), cleaving or cutting it or changing its orientation in such a way that it can no longer work as a signal molecule for the process of quorum quenching. As shown in Fig. 1.10, three types of enzymes have been reported to act on AHLs and can be termed as quorum quenching enzymes (Utari et al., 2017).

i) AHL lactonase- AHL lactonase hydrolyzes the ester bond of the homoserine lactone ring of acylated homoserine lactones, cleaving the lactone ring. As the structure of the AHL molecule is changed, it is unable to act as a signal molecule since it cannot bind to its target transcriptional regulators, thus inhibiting quorum sensing. AHL lactonases enzymes found in QQ bacteria belong to various different protein families such as Metallo-β-lactamase-like lactones, Phosphotriesterase-like lactones (PLLs) and Paraoxonases (PONs). Metallo-β-lactamase-like lactones super-family consists of a Zn^{2+}-binding HXHXDH motif and is present in AiiA, AttM/AiiB, AhlD, AhlS, AidC, QlcA etc. lactonases. PLLs belong to the amidohydrolase superfamily, containing a binuclear metal center within a $(\beta/\alpha)_8$-barrel structural scaffold. They work against a broad spectrum of AHLs but are known to prefer hydrophobic lactones. QsdA, GKL, GsP etc. lactonases belong to this super-family. Usually, mammalian lactonases belong to the Paraoxonases family. They have a structural and catalytic Ca^{2+} ion and a six-bladed β propeller fold. (Fetzner, 2015). AdeH, an AHL lactonase cloned from *Lysinibacillus* sp. in *E. coli* in our laboratory was found to be a unique one as it shows similarity to lactonases belonging to metallo-β-lactamase superfamily as well as the amidohydrolase superfamily. AdeH varies in the way that most *Bacillus* isolates demonstration the occurrence of lactonase AiiA, while AdeH demonstrates only 30% resemblance to AiiA (Garge & Nerurkar, 2016).

ii) AHL acylase- AHL acylases also called amidase or aminohydrolase catalyzes and cleaves N-acyl homoserine lactone into homoserine lactone and an acyl side chain. The cleavage of the AHL molecule, inactivates its function as signal molecule and inhibits quorum sensing in the bacteria. Unlike AHL lactonases, AHL acylases belong to a single protein superfamily consisting of a N-terminal nucleophile hydrolase fold

with an exception of AiiO from *Ochrobactrum* sp. which has a α/β hydrolase fold (Czajkowski et al., 2011a). AHL acylases have been reported to have a preference to long-chain AHLs (Fetzner, 2015; Utari et al., 2017). AHL acylase has been purified from *Delftia* sp. VM4 which shows antagonism against *Pcc*BR1 (Maisuria & Nerurkar, 2015).

iii) AHL oxido-reductase – AHL oxidoreductase do not degrade the AHL molecule like lactonases or acylases but they simply substitute the oxo-group at C3 carbon position with a hydroxyl group, which is degraded easily by amidohydrolases (Chen et al., 2013).

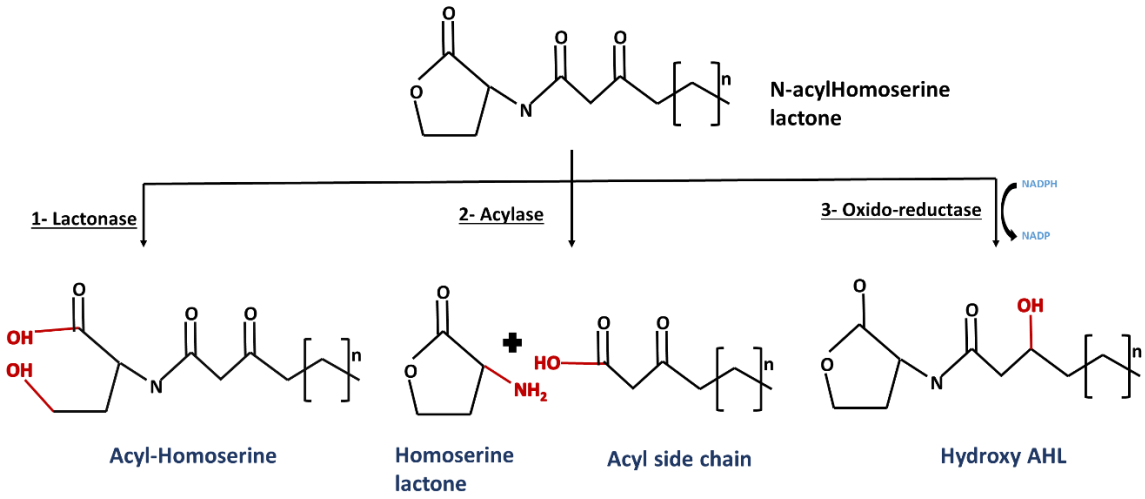

Figure 1.10 Major classes of AHL inactivating enzymes produced by bacteria.

Several bacteria have been reported to be producing the quorum quenching enzymes. Fetzner, (2015) have provided in their studies a comprehensive list of QQ enzymes and the protein family to which they belong. (Koul & Kalia, 2017) have performed a comparative genomic analysis and expressed that there exists multiplicity of genes for AHL lactonases and acylases which emphasizes that it is a potential mechanism that limits the target bacterial population.

1.6.3 Actinomycetota as biocontrol against phytopathogens

Actinomycetota is an interesting phylum with regard to its quorum sensing and quorum quenching potential. *Arthrobacter* sp. was found to grow on 3-oxo-C6-AHL and degraded several other AHLs with diverse lengths. It reduced the virulence by

reducing the AHL and pectate lyase when grown together in a co-culture with soft rot causing *Pcc*. It has an AHL lactonase gene, AhlD, which has 25%, 26% and 21% similar identity to other known AHL-degrading enzymes, viz. *Bacillus* sp. 240B1 AiiA, a *Bacillus thuringiensis* subsp. *kyushuensis* AiiA homologue and *Agrobacterium tumefaciens* AttM (Park et al., 2003).

Microbacterium testaceum, isolated from leaf surface of potato showed presence of AHL-degradation activity and the gene responsible for AHL degradation was AiiM, which was a part of α/β hydrolase fold family of *Actionobacteria.* AiiM was found out to be an AHL lactonase and it was used against phytopathogen *Pcc* where it reduced the pectinase activity and the soft rot symptoms in potato *in vitro* (Wang et al., 2010).

Rhodococcus erythropolis is another widely used bacteria belonging to phylum *Actinomycetota* as a quorum quencher against QS phytopathogens. *R. erythropolis* is an exceptional bacterium in which all three AHL degrading enzymes AHL lactonase, acylase and oxido-reductase have been reported. It encodes a unique class of quorum quenching lactonases which do not show similarity with lactonases which have been previously reported like AiiA, AhlD etc. The AHL lactonase gene QsdA (**Q**uorum-sensing **s**ignal **d**egradation) degrades a wide range of AHLs and this gene is related to phosphotriesterases (Uroz et al., 2008; Cirou et al., 2012). Apart from the AHL lactonase activity by qsdA gene in *R. erythropolis* specific genes for AHL acylase and oxido-reductase have not been identified yet, however vast range of AHL signal molecules have been reported to be degraded by these two types of enzymes in *R. erythropolis* (Uroz et al., 2005). Bio-stimulated by ɣ-caprolactone and isolated from potato plants cultivated in hydroponic conditions, AHL degrading *R. erythropolis* was isolated and its efficiency as a quorum quencher against *Pectobacterium* was found to be good (Cirou et al., 2011). Further studies on their quorum quenching activity against Gram negative soft-rot causing plant pathogens, by degrading their signal molecules AHL have provided an idea on the ɣ-lactone catabolic pathway. ɣ-lactone catabolic pathway is accountable for cleaving of the lactone ring in the AHL molecule which is linked to an alkyl or acyl chain. This pathway is controlled by the availability of ɣ-lactone, therefore, stimulating it with food flavoring like ɣ-caprolactone increased the biocontrol potential of *R. erythropolis* and this was shown to promote plant protection *in vivo* (Latour et al., 2013).

Streptomyces sp. has also been reported to have an AHL degrading enzyme, an acylase which plays a role in keeping virulence factors of pathogens in check. *Streptomyces* sp. which is found in abundance in soil can indeed be a useful biocontrol agent against phytopathogens. It shows the presence of AhlM gene which is an AHL acylase and shows minor similarities to AHL acylases of *Ralstonia* (AiiD) and *Pseudomonas aeruginosa* (PvdQ). The AhlM has been able to reduce the QS based virulence factors like elastase, protease. The gene LasA in *P. aeruginosa* decreased its pathogenicity and making it an important biocontrol agent to attenuate or completely reduce the AHL-based pathogenicity (Park et al., 2005).

A more detailed review on the quorum sensing ability of Actinomycetota and its quorum quenching ability has been reported by (Polkade et al., 2016). Thus, Actinomycetota has been an important phylum having some very effective quorum quenching bacteria which work against gram negative based plant pathogens using quorum sensing for their pathogenicity.