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N-acyl-homoserine lactone-mediated quorum sensing blockage, a novel strategy for attenuating pathogenicity of Gram-negative bacterial plant pathogens

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Abstract

Quorum sensing is a bacterial communication mechanism by which bacteria sense their own population size and couple specific gene expression to cell density. In Gram-negative bacteria, the most commonly used quorum sensing signals are *N*-acyl homoserine lactones (AHLs). It is now apparent that many pathogenic bacteria employ quorum sensing to control premature expression of virulence factors. This control is thought to decrease the likelihood that the plant host would detect the pathogen's presence and activate its defense system. Novel strategies that target bacterial quorum sensing systems in order to control plant bacterial diseases are discussed.

Abbreviations: ACP = acyl carrier protein; AHL = *N*-acyl-homoserine lactone; EPS = extracellular polysaccharide; HPLC = high pressure liquid chromatography; HSL = homoserine lactone; MS = mass spectrometry.

Introduction

Recent advances in studies of bacterial gene expression have shown that many bacteria employ a dedicated inter-cellular communication system. This bacterial decision-making system enables an individual bacterium to sense, integrate and process information from its surroundings, communicate with each other, and monitor its own population density and, as a response, activate or repress specific gene expression. This bacterial cell density-dependent communication system is known as quorum sensing (Fuqua et al., 1994).

To sense the surrounding bacterial population density, the bacterial quorum sensing system relies on one or more small signal molecules (also called 'autoinducers'), which are produced and released by bacteria, and the cognate autoinducer receptor proteins (LuxR homologues or R protein) (Figure 1). LuxR homologues can function as a repressor or activator, depending on the bacterial species. In Gram-negative bacteria, the most commonly utilized and intensively investigated autoinducers are *N*-acyl-homoserine lactones (AHLs) (Figure 2). The acyl side-chain length and the substitutions on the side chain provide signal specificity. So far, AHLs with side chain lengths of 4–18 carbons have been identified (Eberhard et al., 1981; Fuqua et al., 1996; Fray, 2002; Schaefer et al., 2002). The AHL synthase (LuxI homologues) and the LuxR homologues (also known as autoinducer receptor proteins or R proteins) are highly conserved at the nucleotide level and AHL-mediated quorum sensing is widespread among Gram-negative bacteria (Bainton et al., 1992a, b; Swift et al., 1993). Individual bacterial cells usually constitutively produce a basal level of the AHL signal, which is not sufficient to activate the

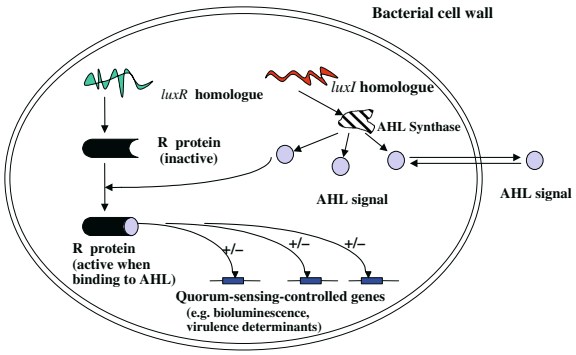
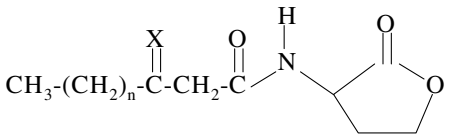
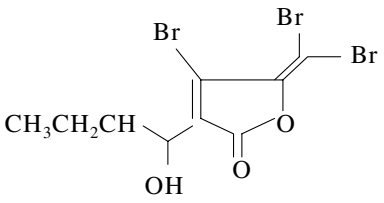


Figure 1. Quorum sensing circuit. Two regulatory genes (*luxR* homologue and *luxI* homologue) are required for quorum sensing controlled gene expression. When the cell population is low, insufficient AHL signals are present to activate the LuxR protein (R protein). As the cell population increases, the concentration of the AHL signals increases both intra- and extracellularly. At a critical concentration, the R protein is activated through binding to the AHL signal. The activated R protein acts as a transcription activator or repressor and therefore regulates the quorum-sensing-controlled gene expression.



AHL signal (X= H,OH or O; n = 2-14)



Halogenated furanone

Figure 2. AHL type quorum sensing signals and halogenated furanone signal mimics.

quorum sensing system. When adequate bacterial cells are present or the environment is non-porous, AHLs reach a threshold concentration and are capable of binding to and activating their cognate receptor proteins, which in turn leads to the activation or derepression of target genes (Figure 1).

Bacteria employ quorum sensing to control a variety of physiological processes, including bioluminescence, swarming, swimming and twitching motility, antibiotic biosynthesis, biofilm differentiation, sporulation, alternative sigma factor synthesis, plasmid conjugal transfer and the production of virulence determinants (Pierson and Thomashow, 1992; Gambello et al., 1993; Piper et al., 1993; Zhang et al., 1993; Pearson et al., 1994, 1995; Beck von Bodman and Farrand, 1995; Cooper et al., 1995; Puskas et al., 1997). This review will focus on quorum sensing-mediated pathogenicity and virulence factor expression in Gram-negative plant pathogenic bacteria.

Quorum sensing controls pathogenicity and virulence gene expression in plant pathogenic bacteria

Although quorum sensing was discovered in the context of marine microbial ecology, it is rapidly becoming clear that the regulation of virulence determinants via quorum sensing in pathogenic bacteria is more the rule than the exception. A common feature of many bacterial infections is the need for the infecting pathogen to reach a critical cell population density, or quorum, high enough to overwhelm host defenses. It therefore makes considerable sense that quorum sensing regulates the production of pathogenicity and virulence factors.

Erwinia carotovora

E. carotovora causes soft rot on a broad range of hosts. The production of several exoenzymes (plant tissue-degrading enzymes), including cellulase, pectinases, polygalacturonase and proteases, mediate pathogenicity in this organism (Collmer and Keen, 1986; Hinton et al., 1989; Barras et al., 1994). A quorum sensing system consisting of an AHL synthase (ExpI) and an autoinducer receptor protein (ExpR) have been discovered to control virulence in *E. carotovora* (Pirhonen et al., 1993). *expI* mutants, deficient in AHL production, are avirulent and show a pleiotropic defect in exoenzyme gene expression (Pirhonen et al., 1991, 1993). Both synthetic AHL or transformed *E. coli* harboring the cloned *luxI* gene (AHL synthase in *V. fischeri*) are capable of restoring the synthesis of exoenzymes and bacterial virulence. Addition of exogenous AHL to the *expI* mutants restored both the production of the plant tissue-macerating exoenzymes and the ability of the bacteria to

propagate *in planta* (Jones et al., 1993; Pirhonen et al., 1993).

Erwinia stewartii

E. stewartii is a plant-pathogenic bacterium that causes Stewart's wilt of sweet corn. It produces an extracellular polysaccharide (EPS) capsule which plays a role in the development of the disease (Braun, 1982; Dolph et al., 1988; Leigh and Coplin, 1992). A quorum sensing system, consisting of a quorum sensing signal synthase EsaI and a LuxR homologue regulatory protein EsaR has been identified in *E. stewartii* (Beck von Bodman and Farrand, 1995). EsaI is responsible for the quorum sensing signal production of 3-oxohexanoyl homoserine lactone. Production of *E. stewartii* AHL is a critical component in fostering the bacterial association with susceptible host plants. Further study discovered that the production of EPS, the key virulence factor of *E. stewartii*, requires an AHL quorum sensing signal and mutants that are deficient in AHL production are less virulent on a susceptible sweet corn genotype (Beck von Bodman and Farrand, 1995).

Agrobacterium tumefaciens

A. tumefaciens is a plant pathogen that induces crown gall tumors on susceptible host plants through a mechanism of trans-kingdom gene transfer. In this mechanism, a segment of plasmid DNA called transfer-DNA (T-DNA) of the tumor-inducing plasmid (pTi) is secreted and transferred to the nucleus of the host plant cell where the T-DNA covalently integrates in the nuclear genome. The integrated T-DNA encodes genes that direct the synthesis of opines and phytohormones, auxin and cytokinin, which leads to uncontrolled proliferation of the host cells that received T-DNA (Sheng and Citovsky, 1996; Christie, 1997).

In *A. tumefaciens*, quorum sensing controls the replication and conjugal transfer of the tumor-inducing (Ti) virulence plasmid. In this quorum sensing system, the QSS was identified as 3-oxo-C_8-homoserine lactone (Zhang and Kerr, 1991; Zhang et al., 1993). Shortly after this, a QSS receptor protein, called TraR, and the QSS synthase, designated TraI, were characterized (Piper et al., 1993; Hwang et al., 1994). The TraR, when binding to 3-oxo-C_8-homoserine lactone, activates the expression of the *tra* regulon (Piper et al., 1993).

Burkholderia cepacia

Burkholderia cepacia can be both a human opportunistic pathogen and a plant pathogen. It was first described as an aggressive plant pathogen responsible for bacterial rot of onions (Burkholder, 1950). Although Lewenza et al. (1999) have identified a quorum sensing system CepI/R and its cognate AHL signal as *N*-octanoyl homoserine lactone in a human pathogenic strain, the quorum sensing in the plant pathogen *B. cepacia* remained elusive until Aguilar et al. (2003) discovered the quorum sensing system from an onion pathogenic strain *B. cepacia* ATCC 25416. The quorum sensing system (*cepI*/*cepR*) regulates protease and polygalacturonase. The *cepI* and *cepR* knockout mutants are attenuated in onion pathogenicity (Aguilar et al., 2003).

Ralstonia solanacearum

Ralstonia solanacearum causes vascular wilt in many different host species including tomato, tobacco and potato (Hayward, 1995). The pathogenicity of *R. solanacearum* is dependent on the production of extracellular polysaccharide (EPS) and plant cell-wall degrading enzymes (Schell, 1996). A LasR-type regulator, PhcA, is responsible for the regulation of virulence factor production. PhcA activity is regulated by an autoinduction system mediated by 3-hydroxypalmitic acid methyl ester [non-AHL-mediated quorum sensing (Clough et al., 1997)]. An AHL-based quorum sensing system (*solR*/*solI*) has been identified in this bacterium, which operates through C_6-HSL and C_8-HSL (Flavier et al., 1997). The AHL-dependent quorum sensing system is part of a more complex autoregulatory hierarchy, since its expression is regulated by a 3-OH PAME-dependent system via PhcA (Flavier et al., 1997).

Pseudomonas aeruginosa

Pseudomonas aeruginosa is well known as a human and animal pathogen. It employs two AHL-mediated quorum sensing systems LasR/I and RhlR/I (Passador et al., 1993; Ochsner and Reiser, 1995). At the genetic level it is now apparent that the

expression of many *P. aeruginosa* virulence determinants are regulated to varying degrees through these quorum sensing systems (Gambello et al., 1993; Williams et al., 1996; Glessner et al., 1999; Pesci and Iglewski, 1999). Rahme et al. (1995) discovered that *P. aeruginosa* (UCBPP-PA14) is also capable of infecting *Arabidopsis thaliana* leaves. *P. aeruginosa* (UCBPP-PA14) mutants in two animal-virulence-related genes, *plc*S (phospholipase S) and *tox*A (exotoxin A), caused attenuated soft-rot and chlorosis symptoms in the *Arabidopsis* model. Furthermore, a *P. aeruginosa* mutant deficient in *gac*A (plant virulence determinant) caused less severe disease not only in *Arabidopsis*, but also in the mouse model. The above results suggested that mechanisms for regulation of virulence factors might be conserved between plant and animal pathogens (Rahme et al., 1995).

Pseudomonas fluorescens

A quorum sensing system MupR/I has been identified in *P. fluorescens* strain NCIMB 10586 (EI-Sayed et al., 2001). The MupR/I controls mupirocin (polyketide antibiotic) production and shows significant identity to other quorum sensing regulatory systems such as LuxR/I in *Vibrio fischeri* and LasR/I in *P. aeruginosa* (EI-Sayed et al., 2001). *P. fluorescens*, although well known as a biocontrol agent, is also an opportunistic field and post-harvest soft-rot pathogen of vegetables. Our recent work has identified an AHL signal in the broccoli pathogen *P. fluorescens* 5064 as *N*-3-hydroxyoctanoyl-homoserine lactone and provided evidence that quorum sensing controls biosurfactant production in this strain (Cui et al., 2004). Biosurfactants and pectolytic enzymes are pathogenicity factors of *P. fluorescens* and play an important role in disease development (Hildebrand, 1989).

Other plant-pathogenic bacteria

Pseudomonas syringae strains produce many AHLs and most of them are short-chain AHLs (Dumenyo et al., 1998; Elasri et al., 2001). No absolute correlation between genomospecies of *P. syringae* and their ability to produce AHL was found (Elasri et al., 2001). So far, there is no evidence to show that pathogenicity in *P. syringae* is correlated to these AHLs. *Xanthomonas campestris* and *Erwinia chrysanthemi* are also reported to exhibit quorum sensing systems based on AHL, but the functions of these quorum sensing systems remain unknown (Matsuyama et al., 1992; Cha et al., 1998).

The benefit of employing a cell density-dependent quorum sensing system in pathogenic bacteria

A potential advantage for employing a density-sensing system is to ensure the bacteria have an economic life style. Usually, a mass of cells is required to produce sufficient quantities of extracellular factors to influence the surrounding environment. It would be a waste of resources if bacteria start to produce these factors before a critical population has been reached (Greenberg, 2000). For plant pathogenic bacteria, the ability to successfully invade specific hosts depends on the evolution of a few gene systems – virulence and pathogenicity genes. These genes are frequently activated in the presence of the plant host, and the products of these activated genes alter the plant host so that disease is induced (Dunny and Winans, 1997). What are the particular advantages of possessing a population-dependent regulatory system for pathogenicity and virulence genes in bacterial pathogens? A major objective of an invading bacterium is the overwhelming of its host's defenses. Therefore, the premature expression of bacterial virulence factors, which could possibly alert the plant host and elicit subsequent defensive responses, would not provide a successful strategy for such pathogens. Quorum sensing provides an effective timing-control mechanism in pathogenicity factor expression and allows the pathogen to amass, without being sensed by the host, until a big enough population is achieved to overwhelm the plant host. In some bacteria, as well as in *V. fischeri*, the quorum sensing system forms a positive feedback circuit, where the AHL synthase is part of the quorum sensing induced regulon. This will allow for rapid signal amplification and thus an accelerated induction of the aggressive phenotype to full potential (Engebrecht et al., 1983; Hwang et al., 1994; Chan et al., 1995; Seed et al., 1995; Winson et al., 1995; Swift et al., 1997; Cui et al., 2004).

In plant pathogens such as *Erwinia carotovora*, the direct relationship between pathogenicity and AHL production indicates the crucial role of quorum sensing in bacterial pathogenicity (Jones et al., 1993; Pirhonen et al., 1993) as AHL deficient mutants of *E. carotovora* are non-pathogenic. However, when the mutant bacteria are pre-incubated with their species-specific AHL and inoculated onto plant leaves, pathogenicity is partially restored. Further tests show AHL is required not only for *E. carotovora* to initiate the infection, but also for the infection to persist (Pirhonen et al., 1993). Once invasion has been successful, other soil and plant-borne bacteria could compete for the suddenly released nutrients from the plant. To minimize this potential disadvantage, *E. carotovora* has adopted a strategy of coordinating the release of a broad-spectrum antibiotic (carbapenem) along with plant-degradative enzymes (Swift et al., 1999a).

Employing the quorum sensing system as a target for novel anti-microbial strategies

The discovery that a wide spectrum of bacteria use quorum sensing to control virulence factor production makes it an attractive target for antimicrobial therapy (Finch et al., 1998). Mechanisms by which quorum sensing may be blocked and disease control achieved are discussed below.

Using AHL analogs as antagonists

In most bacterial quorum sensing systems, the AHLs and their cognate R receptor proteins (LuxR homologues) have a high affinity for one another. Non-cognate signals–R protein complexes might have the ability to activate the quorum sensing-related genes weakly. However, more often, non-cognate AHLs act as antagonists, bind to but do not activate R proteins (Eberhard et al., 1986; Hanzelka and Greenberg, 1995; Pearson et al., 1995; Zhu et al., 1998). The possibilities of using AHL analogs to attenuate quorum sensing regulation systems have been explored in *V. fischeri, Aeromonas hydrophila* and *A. salmonicida, A. tumefaciens, E. carotovora* and *Chromobacterium violaceum* (Eberhard et al., 1986; Chhabra et al., 1993; Hanzelka and Greenberg, 1995; Schaefer et al., 1996b; Swift et al., 1997, 1999b; Zhu et al., 1998). For example, in *A. hydrophila,* the C_4-HSL-dependent exoprotease production can be virtually abolished by the addition of AHL with acyl side chains of 10, 12 or 14 carbons (Swift et al., 1999b).

Givskov et al. (1996) were the first to discover that a eukaryote, the marine red macro-alga *Delisea pulchra*, secretes compounds that act as mimics of bacterial AHL-type quorum sensing signals. These AHL signal mimics are halogenated furanones (Figure 2) and are structurally similar to AHLs and inhibit quorum sensing regulation in various bacteria (de Nys et al., 1993; Givskov et al., 1996). *Delisea pulchra* furanones have been found to inhibit AHL-regulated processes, including swarming in *Serratia liquefaciens,* virulence of *V. harveyi*, bioluminescence in *V. fischeri* and antibiotic and exoenzyme production in *E. carotovora* (Givskov et al., 1996; Kjelleberg et al., 1997; Srinivasan et al., 1998; Rasmussen et al., 2000; Manefield et al., 2000, 2001). As the inhibitory effect can be overcome by the addition of an excess of the cognate AHL, the furanones appear to be acting as competitive antagonists. But recently, Manefield et al.'s work (Manefield et al., 2002) revealed that halogenated furanones modulate LuxR activity through accelerating LuxR turnover, rather than protecting the AHL-dependent transcriptional activator. They suggest that the reduction in LuxR concentration is the mechanism by which furanones control expression of AHL-dependent phenotypes.

In addition to the halogenated furanones of *D. pulchra*, a variety of bacteria and eukaryotes have been shown to produce cyclic dipeptides (diketopiperazines) that can act as AHL mimics to affect quorum sensing-regulated behaviors in bacteria (Holden et al., 1999). The unicellular soil and freshwater alga *Chlamydomonas reinhardtii* was also found to secrete AHL mimic substances (Teplitski et al., 2004). The purified *C. reinhardtii* mimic affected the soil bacterium *Sinorhizobium meliloti* quorum sensing-regulated gene expression and was able to cancel the stimulatory effects of the AHL produced by *S. meliloti*. This provides evidence that the secretion of AHL mimics by the alga could be effective in disruption of quorum sensing in naturally encountered bacteria (Teplitski et al., 2004).

More recently, various higher plants were also shown to secrete AHL signal mimic substances

that are capable of interfering with bacterial quorum sensing (Teplitski et al. 2000; Daniels et al., 2002; Gao et al., 2003; Mathesius et al., 2003). Although the chemical nature of the active compounds from plants and their effect on bacterial virulence are not clear, these AHL signal mimic substances, through interfering with bacterial quorum sensing, could be one of the intrinsic plant defense mechanisms against pathogenic invaders.

Using AHL analogs as antagonists to interfere with quorum-sensing-dependent bacteria might be used as a strategy in controlling bacterial pathogens. However, it is essential to determine whether they can function *in vivo* at a physiologically relevant concentration. For successful application in conventional breeding or transgenic approaches, the genes involved in the biosynthesis of these AHL analogs need to be identified and cloned, and this remains a challenging task (Bauer and Teplitski, 2001).

Quorum sensing signal degradation

Many acyl-HSLs are stable in mildly acidic or neutral pH environments. However, there is no evidence that they accumulate in such environments. If they accumulated over long periods of time, their function as quorum sensing signals would be disarmed and signal concentration would not reflect cell number after fluctuations in population density. Furthermore, as AHL-producing bacteria are ubiquitously present in natural environments, it can be expected that other organisms have evolved means to interfere with this type of communication. The above facts imply that a possible natural acyl-HSL signal degradation pathway exists.

Dong et al. (2000) discovered an enzyme from *Bacillus* sp. 240 B1 that is capable of degrading AHL. This enzyme is encoded by the *aiiA* gene. The enzyme inactivates AHL activity by hydrolyzing the lactone bond of AHL and is named AHL lactonase (Dong et al., 2001). Later studies discovered that the AHL-degrading enzyme-encoding genes are widespread in many subspecies of *Bacillus*. Lee et al. (2002) studied the *aiiA* homologue genes in 16 subspecies of *B. thuringiensis* genome sequences. When compared with the *Bacillus* sp. 240 B1 *aiiA* gene (Dong et al., 2000), these *aiiA* showed high homologies of 89–95% in the nucleotide sequences and 90–96% in the deduced amino acid sequences. These results indicated that insecticidal *B. thuringiensis* strains might have the potential to compete with Gram-negative bacteria in natural ecosystems by autoinducer-degrading activity. Apart from these *B. thuringiensis* species, two closely related species *B. cereus* and *B. mycoides* were shown to produce AHL-inactivating enzymes, while *B. fusiformis* and *B. sphaericus* strains do not (Dong et al., 2002). Expression of *aiiA* in the transformed *E. carotovora* strain SCG, a pathogen that causes soft rot disease in many plants, significantly reduces release of AHL, decreases extracellular pectolytic enzyme activities, and attenuates pathogenicity in potato, egg-plant, Chinese cabbage, carrot, celery, cauliflower and tobacco (Dong et al., 2000). In a recent study, Dong et al. (2004) discovered that *B. thuringiensis*, when coincubated with *E. carotovora*, abolished its accumulation of AHL signal and significantly decreased the symptom development of potato soft rot caused by this pathogen. Transgenic plants expressing AHL lactonase exhibit significantly enhanced resistance to *E. carotovora* infection and delay development of soft rot symptoms (Dong et al., 2001).

A motile, rod-shaped soil bacterium, *Variovorax paradoxus* has been shown to degrade and utilize *N*-(3-oxohexanoyl)-*L*-homoserine lactone as the sole source of energy and nitrogen (Leadbetter and Greenberg, 2000). Further tests showed that the *V. paradoxus* isolate is capable of growth on all of the acyl-HSLs tested. From the same enriched culture that yielded *V. paradoxus*, Flagan et al. (2003) discovered that an *Arthrobacter* strain VAI-A, though not capable of degradation of AHLs, could utilize the nitrogenous degradation products of AHLs as sources of energy and nitrogen. They therefore hypothesized that the original co-enrichment of these two organisms from the same soil sample was not coincidental and that the consortium might play a role in quorum sensing turnover (Flagan et al., 2003).

Recently, Lin et al. (2003) cloned a gene, named *aiiD*, that is capable of encoding a novel and potent AHL acylase in a *Ralstonia* isolate. The gene product (AiiD) is a polypeptide of 794 amino acids. Sequence homology searches indicate that AHL acylase is conserved in many different bacterial species. HPLC and MS analysis demonstrated that AiiD hydrolyzes the AHL amide, releasing homoserine lactone and the corresponding fatty acid. AiiD has been proved to be a potent enzyme;

expression of AiiD in *Pseudomonas aeruginosa* PAO1 quenched quorum sensing in this bacterium, decreasing its ability to swarm, produce elastase and pyocyanin and to paralyze nematodes (Lin et al., 2003).

Uroz et al. (2003) reported the isolation of several bacteria that are capable of degrading AHLs, all with different specificity and kinetics. One of these isolates, *Rhodococcus erythropolis* W2, can interfere with the violacein production of *C. violaceum* and the pathogenicity of *A. tumefaciens* when co-cultured together. *In planta*, *R. erythropolis* significantly reduces the pathogenicity of *Pectobacterium* (*Erwinia*) *carotovora* subsp. *carotovora* in potato tubers (Uroz et al., 2003).

Harnessing AHL degradation by applying to plants biocontrol agents that produce AHL-degrading enzymes, could be used to control specific plant diseases. The required specificity would be achieved through the use of degradative enzymes targeted at particular AHLs.

Interrupting the quorum sensing signal biosynthetic pathway and global repressor genes

Two biosynthetic pathways related to AHL synthesis have been elucidated. One is the fatty acid biosynthesis pathway, by which the acyl side chain is synthesized. Another is the synthesis of homoserine lactone from S-adenosylmethionine (Hanzelka and Greenberg, 1996; More et al., 1996; Schaefer et al., 1996a; Jiang et al., 1998). Enoyl-ACP reductase is an important enzyme in AHL biosynthesis. An enoyl-ACP reductase inhibitor, triclosan, has been reported that is capable of reducing AHL production *in vitro* (Hoang and Schweizer, 1999).

Interrupting the AHL biosynthetic pathway and shutting down AHL synthesis, perhaps through the use of analogs of AHL precursors or inhibition of the enzymes that are necessary for the synthesis, would be a highly effective means of blocking the quorum sensing cascade (de Kievit and Iglewski, 2000).

The recent significant advances in defining the enzymatic activities and substrate requirements of *luxI* homologues emphasize the potential of using the AHL synthase as an antimicrobial target (More et al., 1996; Schaefer et al., 1996a; Jiang et al., 1998; Parsek et al., 1999). So far, several global repressor genes have been found to reduce the levels of transcripts of *luxI* homologues. Branny et al. (2001) have isolated a multi-copy suppressor gene *dksA* from *P. aeruginosa*, which is a homologue to the *E. coli dnaK*. Over-production of this *P. aeruginosa dksA* gene inhibits quorum sensing-dependent virulence factor production by down-regulating the transcription of the autoinducer synthase gene *rhlI*. Another global repressor gene, *qscR*, has recently been described as a modulator of quorum sensing signal synthesis and virulence in *P. aeruginosa* (Chugani et al., 2001). The *qscR* gene product governs the timing of quorum sensing controlled gene expression. Its primary role is to repress *lasI*, an AHL synthase gene in *P. aeruginosa*. The repression of *lasI* by QscR could serve to ensure that quorum-sensing-controlled genes are not activated in environments where they are not useful. A *qscR* mutant produces the *lasI*-generated signal prematurely, and this results in premature transcription of a number of quorum sensing-regulated genes.

In addition to *qscR* and *dksA*, other global repressor genes such as *rsaL*, which is located downstream from LasR in *P. aeruginosa* (de Kievit et al., 1999) and *rsmA*, which was identified in *E. carotovora* subsp. *carotovora* T_1 mutant (Cui et al., 1995), have also been reported. Overproduction of these AHL synthase repressor genes in either plant pathogenic bacteria or biocontrol agents would be an attractive strategy in quenching quorum-sensing-dependent virulence factor production.

Interference with the bacterial membrane multi-drug efflux pump

Originally described in bacteria, efflux pumps (drug transporters) are now recognized as common membrane components in all cell types, from prokaroytes to eukaryotes (van Bambeke et al., 2003). They confer to bacteria a common and basic mechanism of resistance to antibiotics by extruding antibiotics or other toxic molecules from the cell (McMurry et al., 1980). In an investigation of whether AHL can diffuse freely in and out of *P. aeruginosa* cells, it was discovered that, in addition to its slow diffusion, 3-oxo-C_{12}-homoserine lactone is actively pumped from cells by the MexAB–OprM pump (Pearson et al., 1999). This finding is interesting because it suggests that antimicrobial therapy designed to interfere with the

bacterial membrane pump and increase the antibiotic susceptibility of pathogenic bacteria will also affect this bacterial quorum sensing-controlled gene expression and thus become more effective (de Kievit and Iglewski, 2000).

Manipulating plants to interfere with pathogenic bacterial quorum sensing

The fact that AHL signals isolated and purified from one bacterium can alter gene expression in a second bacterium *in vitro* (Greenberg et al., 1979; McKenney et al., 1995) has raised the question whether a plant can be engineered to produce AHLs and these AHLs can alter bacterial gene expression. Fray et al. (1999) have taken a first step in engineering plants to interact with bacteria in this way. They constructed a vector that fused the *Yersinia enterocolitica* AHL synthase gene *yenI* to the *Petunia rbcS* ribulose bisphosphate chloroplast-targeting sequence. This translational fusion (contains a translational enhancer sequence from AMV cloned from pBI526) was placed under the control of the 35S CaMV promoter and transformed into tobacco. AHL was isolated from homogenized transgenic plant tissue and the quantity produced was sufficient to induce target gene expression in several recombinant bacterial biosensors and to restore the biocontrol activity of an AHL-deficient *P. aureofaciens* strain. Because tobacco is not a normal host for *E. carotovora*, they created transgenic *yenI*-expressing potato lines in order to test whether small inocula of wild type of *E. carotovora* could be induced to attempt a pathogenic attack prematurely on AHL-producing potato plants, and whether such an infection would result in increased resistance. Surprisingly, the plants proved to be susceptible at inoculum levels as low as 10^2, levels that in an untransformed plant did not cause disease symptoms (Fray, 2002). Fray gave two possible reasons in explaining why *Erwinia* does not normally attempt an infection at lower cell densities even if such an infection is likely to be successful. Firstly, bacteria set a higher quorum sensing threshold to maximize their chances of success in attacking different plant hosts; Secondly, plants often employ strategies to interfere with the bacterial signaling system. Such interference, including production of signal mimics, signal blockers, signal-degrading enzymes or AHL synthase repressors, could prevent bacteria from initiating a pathogenic attack even when a big enough population size has been reached (Fray, 2002). Contradictory results were also obtained by Mae et al. (2001). They observed that the transgenic tobacco carrying the *expI* gene could, on the one hand, partially complement the avirulent phenotype of an *expI*-negative *E. carotovora* mutant and, on the other hand, exhibit enhanced resistance to infection caused by wild type *E. carotovora*. These results imply that the quorum sensing mechanisms in different phytopathogens might vary and the bacteria might have adapted different threshold settings for quorum sensing-related gene activation. Extra care should be taken to account for specific host–pathogen interactions when attempting to attenuate bacterial pathogenicity by this approach (Fray, 2002; Zhang, 2003). Alternatively, Fray (2002) suggests that supplying transgenic plants with the ability to block or degrade AHL signals may provide a more direct approach for engineering resistance to *E. carotovora* rather than giving plants the ability to produce AHL themselves (and thus induce premature pathogenicity expression). As described earlier, Dong et al. (2001) transformed a copy of *aiiA* (AHL degrading) gene into tobacco and potato. The transgenic plants showed significantly enhanced resistance to *E. carotovora*.

Concluding remarks

N-acyl homoserine lactone-mediated quorum sensing plays a critical role in plant–microbe interactions: many pathogenic bacteria employ quorum sensing to regulate their pathogenicity and virulence factor production. In plant pathology, there is major interest to find natural or synthetic compounds, active in small quantities, that are capable of interfering with quorum sensing in pathogenic bacteria, in order to disrupt their pathogenicity/virulence factor production. Bacterial diseases are much more difficult to control than fungal diseases because of the lack of effective and benign plant protection products.

Using quorum sensing as a target to control plant diseases is potentially an attractive strategy. However, plant–microbe interactions are very complicated and comprehensive considerations are essential in order to make the therapeutic strategy

most effective and sustainable. For example, pathogenic *Pseudomonas fluorescens* 5064 employs quorum sensing to control its biosurfactant production, a pathogenicity factor that plays a role in broccoli head rot disease development (Hildebrand, 1989; Cui et al., 2004). The AHL degrading strain *Bacillus* sp. A24 is capable of abolishing *P. fluorescens* 5064 AHL production but is unable to reduce disease severity. Further tests found that *Bacillus* sp. A24 itself is a potent biosurfactant producer and this will mask the AHL degradation effect (Cui, 2004). Furthermore, it is not unusual for bacteria to employ two or more quorum sensing systems. It might be necessary to disarm all of these systems to achieve the expected result. To be successful, the engineering of plants to produce specific quorum sensing mimics to affect specific bacterial pathogens must consider all quorum sensing systems of a particular bacterium. However, the usefulness of treating or engineering eukaryotic hosts to stimulate or alter quorum sensing in associated bacteria will probably depend on the specific host and pathogen combination because, as we mentioned earlier, the quorum sensing regulation system might vary between different host/pathogen systems. However, this specificity itself is an attractive feature, since it allows specific targeting of disease control measures.

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