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Review

Intercellular and intracellular signalling systems that globally control the expression of virulence genes in plant pathogenic bacteria

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SUMMARY

Plant pathogenic bacteria utilize complex signalling systems to control the expression of virulence genes at the cellular level and within populations. Quorum sensing (QS), an important intercellular communication mechanism, is mediated by different types of small molecules, including *N*-acyl homoserine lactones (AHLs), fatty acids and small proteins. AHL-mediated signalling systems dependent on the LuxI and LuxR family proteins play critical roles in the virulence of a wide range of Gram-negative plant pathogenic bacteria belonging to the Alphaproteobacteria, Betaproteobacteria and Gammaproteobacteria. *Xanthomonas* spp. and *Xylella fastidiosa*, members of the Gammaproteobacteria, however, possess QS systems that are mediated by fatty acid-type diffusible signal factors (DSFs). Recent studies have demonstrated that Ax21, a 194-amino-acid protein in *Xanthomonas oryzae* pv. *oryzae*, plays dual functions in activating a rice innate immune pathway through binding to the rice XA21 pattern recognition receptor and in regulating bacterial virulence and biofilm formation as a QS signal molecule. In xanthomonads, DSF-mediated QS systems are connected with the signalling pathways mediated by cyclic diguanosine monophosphate (c-di-GMP), which functions as a second messenger for the control of virulence gene expression in these bacterial pathogens.

INTRODUCTION

A diverse range of virulence factors enable plant pathogenic bacteria to overcome host defence systems, including both pre-existing and induced barriers, during the process of infection and colonization in host plants. For successful infection and multiplication, as well as survival during various environmental conditions throughout a life cycle, plant pathogenic bacteria must be able to control the production of virulence factors in response to dynamic changes in the surrounding environment and their interactions with other members of the microbial community and host plants. Failure to regulate precisely the production of virulence factors to avoid excessive production at inappropriate times would compromise the ecological fitness of the bacterial pathogen through the wastage of the cellular resources necessary for survival, as well as by early detection by the host and consequent activation of host defence systems. In nature, bacteria operate complex systems for signal perception, transduction and exchange at the cellular level and within populations. These signalling systems also play pivotal roles in the fine tuning of the production of virulence factors in pathogenic bacteria.

In this review, bacterial signalling and communication systems utilized by plant pathogenic bacteria are discussed, with a focus on their regulatory functions in bacterial pathogenesis in plants. Intercellular and intracellular signalling systems mediated by different types of signal molecule are described in terms of their functional mechanisms and roles in bacterial virulence. In particular, quorum-sensing systems based on *N*-acyl homoserine lactones (AHLs), diffusible signal factors and Ax21 family small proteins are highlighted, as well as recent advancements in cyclic diguanosine monophosphate (c-di-GMP)-based signalling systems.

INTERCELLULAR SIGNALLING (QUORUM-SENSING) SYSTEMS

Quorum sensing (QS) is an important cell–cell communication mechanism that enables a bacterial population to control the expression of genes primarily based on its density or growth stage. In pathogenic bacteria, QS increases the propensity for successful infection in various hosts as a result of the coordinated expression of virulence genes in accordance with bacterial population levels. Different types of signal molecule are utilized by different bacterial pathogens for QS. The major groups of known QS signal molecules include AHLs, oligopeptides and autoinducer-2 (AI-2) molecules (Roy *et al*., 2011). AHLs are major QS signal molecules in many Gram-negative bacteria, whereas oligopeptides are more commonly used in Gram-positive bacteria. AI-2, a furanosyl borate diester (Cao and Meighen, 1989), is considered to be 'a universal language' in the bacterial world because it is found in both Gram-negative and Gram-positive bacteria (Bassler and Losick, 2006). In plant pathogenic *Xanthomonas* spp. and *Xylella fastidiosa*, the

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primary QS signal molecules are unsaturated fatty acids, called diffusible signal factors (DSFs) (Deng *et al*., 2011). A new type of QS signal, the small protein Ax21, has been discovered recently in these bacteria (Han *et al*., 2011a, 2011b; Ronald, 2011).

QS systems mediated by AHLs

Bacterial QS systems mediated by AHL-type signal compounds are typically composed of two major genetic elements that are homologous to *luxI* and *luxR* of *Vibrio fischeri*, the first QS genes identified and characterized (Fuqua *et al*., 1994). The primary function of the LuxI/LuxR QS system encoded by *luxI* and *luxR* is the autoinduction of luminescence in the marine bacterium *V. fischeri*, in which genes for luminescence are expressed in a cell density-dependent manner (Fuqua *et al*., 1994). LuxI family proteins encoded by *luxI* homologues are AHL synthases, which are responsible for the biosynthesis of AHL molecules, whereas LuxR family proteins encoded by *luxR* homologues are AHL receptor proteins, which are activated by binding to AHL molecules (Fuqua *et al*., 1994; Tsai and Winans, 2010). QS mediated by AHL signal compounds is present in a wide range of Gram-negative bacteria, including many plant and animal pathogens. Here, three representative AHL-mediated QS systems are described in detail. These and other similar QS systems in plant pathogenic bacteria are summarized in Table 1.

TraI/TraR of Agrobacterium tumefaciens

QS systems of *A. tumefaciens* mediated by the *traI* AHL synthase genes and the *traR* AHL receptor genes have been relatively well studied compared with other QS systems of plant pathogenic bacteria. The LuxI family protein, TraI, synthesizes *N*-3-oxooctanoyl-L-homoserine lactone (OOHL), and the LuxR family protein, TraR, is activated by binding to OOHL (Hwang *et al*., 1994; More *et al*., 1996; Zhang *et al*., 1993). OOHL molecules are accumulated at a high cell density and, at a threshold level, TraR-OOHL activates target genes, including the *tra* genes required for Ti plasmid conjugation (Fuqua and Winans, 1994, 1996). TraR is unstable in the absence of the ligand (OOHL) and functions as a homodimer by binding directly to specific DNA sequences, called *tra* boxes (Vannini *et al*., 2002; Zhang R. G. *et al*., 2002; Zhu and Winans, 1999).

TraI/TraR QS systems are under the control of sophisticated fine-tuning systems involving *traM*, *trlR*, *attM* and *attJ*. *traM* is located downstream of *traR* and TraM acts as a negative regulator for TraR activity on the conjugal transfer of Ti plasmids (Fuqua *et al*., 1995; Hwang *et al*., 1995). The inhibitory activity of TraM on TraR may be through direct protein–protein interaction with TraR (Hwang *et al*., 1999). *trlR* encodes a TraR homologue that lacks the C-terminal DNA-binding domain; this TrlR protein is thought to inhibit the activity of TraR on octopine-type Ti plasmids through the formation of inactive heterodimers with TraR (Chai *et al*., 2001; Oger *et al*., 1998; Zhu and Winans, 1998). Because TrlR requires OOHL for solubility, TrlR may function as a TrlR–OOHL complex, like TraR (Chai *et al*., 2001) (Fig. 1).

Meanwhile, TraI/TraR QS systems are also modulated via sophisticated pathways for QS signal turnover. Zhang H. B. *et al*. (2002) found that an AHL-lactonase encoded by *attM* degrades OOHL

Table 1 Representative *N*-acyl homoserine lactone (AHL)-mediated quorum-sensing (QS) systems present in plant pathogenic bacteria.

Bacterium	Gene names for *luxI/luxR* homologues	Primary AHL compounds for QS	Functions	References
Agrobacterium tumefaciens	*traI/traR*	*N*-3-oxooctanoyl-L-homoserine lactone	Conjugal transfer of Ti plasmid	(Fuqua and Winans, 1994; Hwang *et al*., 1994; More *et al*., 1996; Zhang *et al*., 1993)
Burkholderia cepacia	*cepI/cepR*	*N*-octanoyl-L-homoserine lactone	Production of protease and polygalacturonase	(Aguilar *et al*., 2003a)
Burkholderia glumae	*tofI/tofR*	*N*-octanoyl-L-homoserine lactone	Production of toxoflavin and lipase; flagellar biogenesis/motility	(Devescovi *et al*., 2007; Kim *et al*., 2004, 2007)
Pantoea ananatis	*eanI/eanR*	*N*-3-oxohexanoyl-L-homoserine lactone	Production of extracellular polysaccharide; biofilm formation; infection of onion leaves	(Morohoshi *et al*., 2007)
Pantoea stewartii	esaI/esaR	*N*-3-oxohexanoyl-L-homoserine lactone	Production of extracellular polysaccharide; biofilm formation; dissemination and specific localization of bacterial cells at the xylem vessel	(von Bodman *et al*., 1998; Koutsoudis *et al*., 2006)
Pectobacterium carotovorum ssp. *atrosepticum*	*expI/virR*	*N*-3-oxohexanoyl-L-homoserine lactone	Production of virulence factors, including extracellular enzymes	(Burr *et al*., 2006; Liu *et al*., 2008)
Pectobacterium carotovorum ssp. *carotovorum*	*carI/carR*	*N*-3-oxohexanoyl-L-homoserine lactone	Production of carbapenem antibiotic	(McGowan *et al*., 2005)
	expI/expR	*N*-3-oxohexanoyl-L-homoserine lactone	Production of virulence factors, including extracellular enzymes	(Jones *et al*., 1993; Pirhonen *et al*., 1993)
Pectobacterium chrysanthemi	*expI/expR*	*N*-3-oxohexanoyl-L-homoserine lactone	Biosynthesis of pectinases	(Reverchon *et al*., 1998)
Pseudomonas syringae pv. *syringae*	*ahlI/ahlR*	*N*-3-oxohexanoyl-L-homoserine lactone	Production of extracellular polysaccharide; motility	(Quinones *et al*., 2004, 2005)

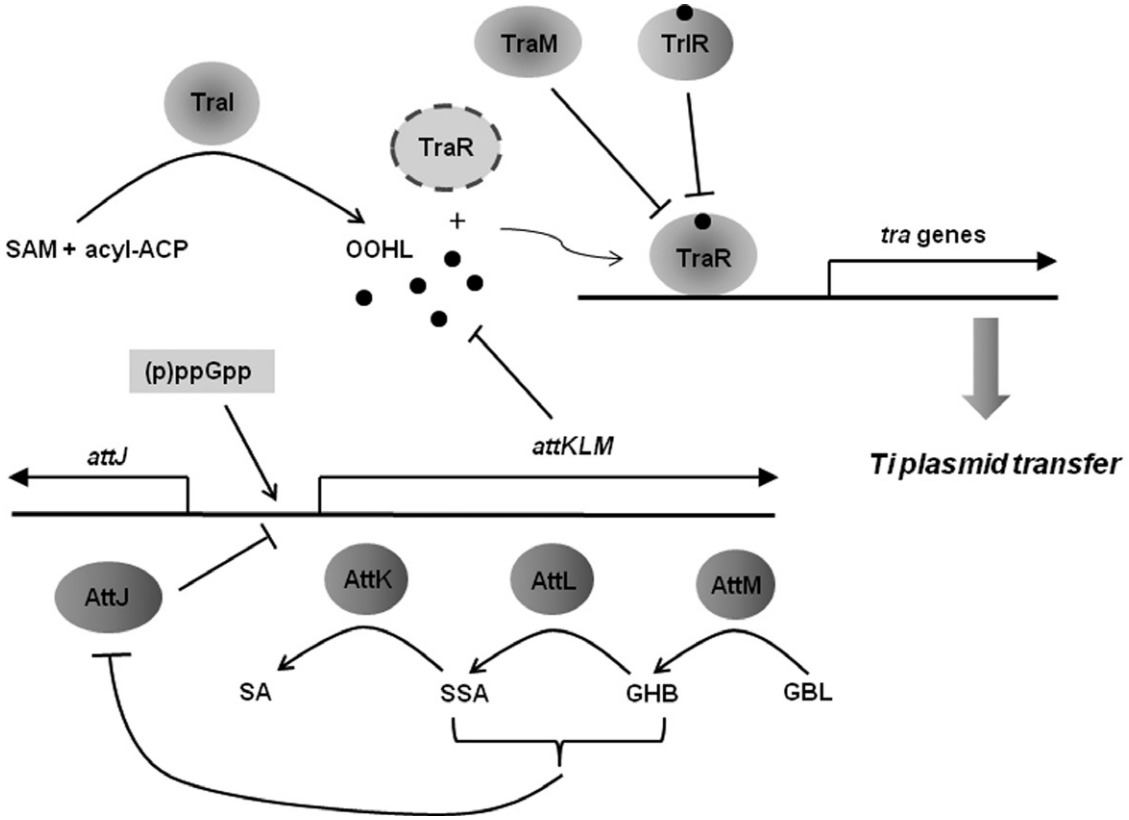

Fig. 1 Schematic view of the TraI/TraR-mediated quorum-sensing system in *Agrobacterium tumefaciens*. TraR alone (depicted by broken line) is unstable. acyl-ACP, acyl-acyl carrier protein; GBL, γ-butyrolactone; GHB, γ-hydroxybutylic acid; OOHL, *N*-3-oxooctanoyl-L-homoserine lactone; SA, succinic acid; SAM, *S*-adenosylmethionine; SSA, succinic acid semialdehyde.

molecules and that the expression of *attM* was repressed by an IclR family protein encoded by *attJ*. Mutation of *attJ* resulted in the constitutive expression of *attM*, which led to the failure of AHL accumulation and conjugal transfer of Ti plasmids (Zhang H. B. *et al*., 2002). *attM* and two adjacent genes, *attK* and *attL*, comprise the *attKLM* operon, and *attJ* is divergently transcribed from this operon (Zhang H. B. *et al*., 2002). *attK* and *attL* encode a succinic acid semialdehyde dehydrogenase and an alcohol dehydrogenase, respectively (Zhang H. B. *et al*., 2002). The *attKLM* operon participates in an assimilative pathway of γ-butyrolactone (GBL) that produces the intermediate metabolites γ-hydroxybutylic acid (GHB), succinic acid semialdehyde (SSA) and succinic acid (SA) through the actions of AttM, AttL and AttK, respectively (Carlier *et al*., 2004; Chai *et al*., 2007). An accepted model for the control of OOHL turnover is that GHB and SSA induce the expression of the *attKLM* operon through the inactivation of the AttJ repressor protein, which, in turn, increases the production of the AttM AHL-lactonase, resulting in the depletion of OOHL and the cessation of Ti plasmid transfer (Carlier *et al*., 2004; Chai *et al*., 2007) (Fig. 1). Degradation of OOHL at the stationary phase, resulting from the expression of *attK*, *attL* and *attM*, is also promoted by either carbon or nitrogen starvation, and dependent on a stress alarmone (p)ppGpp synthase encoded by *relA*, indicating that the degradation of the QS signal is regulated by the stress response machinery involving alarmone (p)ppGpp (Zhang *et al*., 2004) (Fig. 1). It has also been demonstrated that both (p)ppGpp and SSA modulate separately the two succinic acid semialdehyde dehydrogenases, AldH and AttK, which presumably control the intracellular levels of SSA and, consequently, determine the QS signal turnover (Wang *et al*., 2006).

TofI/TofR of Burkholderia glumae

QS mediated by LuxI/LuxR homologues and AHL-type signal molecules is a major regulatory mechanism that controls the production of various virulence factors in *Burkholderia* spp. pathogenic to plants and animals (Aguilar *et al*., 2003b; Devescovi *et al*., 2007; Eberl, 2006; Goo *et al*., 2010; Huber *et al*., 2004; Malott *et al*., 2009; Ulrich *et al*., 2004). It also controls antifungal activities (Schmidt *et al*., 2009), antibiotic production (Duerkop *et al*., 2009; Seyedsayamdost *et al*., 2010) and biofilm formation (Aguilar *et al*., 2003b) in beneficial *Burkholderia* spp. The LuxI homologues of *Burkholderia* spp., represented by the AHL synthase CepI of the *Burkholderia cepacia* complex, mostly catalyse the synthesis of *N*-octanoyl-L-homoserine lactone (OHL) and *N*-hexanoyl-L-

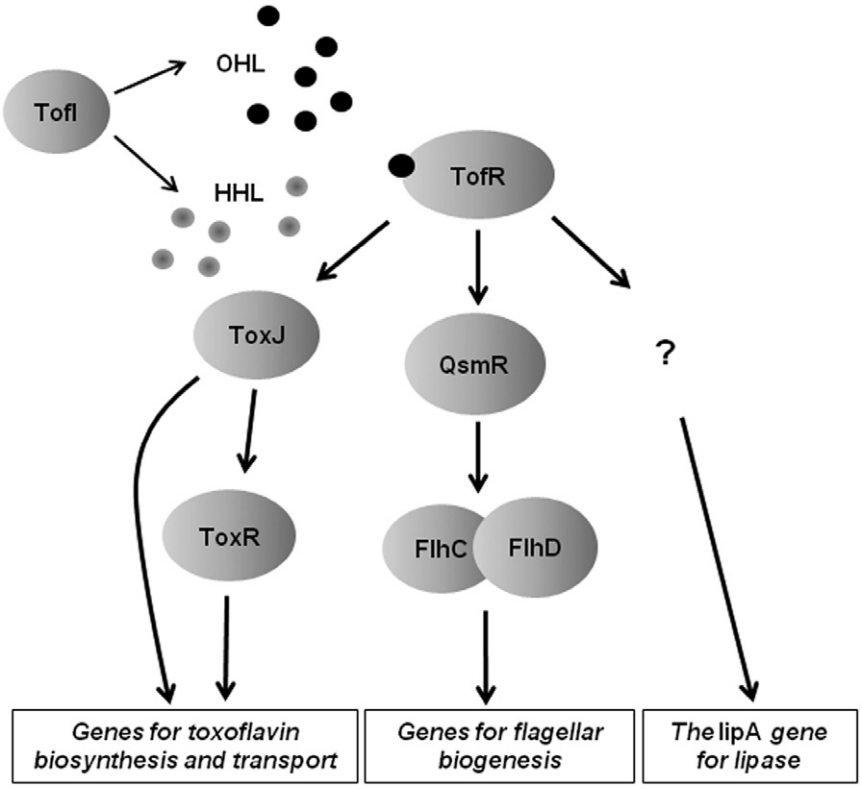

Fig. 2 Schematic view of the global regulatory system mediated by the TofI/TofR quorum-sensing system for the virulence of *Burkholderia glumae*. HHL, *N*-hexanoyl-L-homoserine lactone; OHL, *N*-octanoyl-L-homoserine lactone.

homoserine lactone (HHL); however, only OHL has shown signalling functions for known LuxI/LuxR-type QS systems of *Burkholderia* spp. (Aguilar *et al*., 2003b; Duerkop *et al*., 2007; Kim *et al*., 2007). Recently, other types of signalling molecules for cell–cell communication, 2-heptyl-4(1H)-quinolone (HHQ) and *cis*-2-dodecenoic acid (BDSF), have been found in *B. pseudomallei* and *B. cenocepacia*, respectively (Deng *et al*., 2009; Diggle *et al*., 2006).

In *B. glumae*, which causes bacterial panicle blight and grain rot of rice, the QS system composed of the *luxI* and *luxR* homologues, *tofI* and *tofR*, respectively, globally controls the production of all known major virulence factors: toxoflavin (Kim *et al*., 2004), lipase (Devescovi *et al*., 2007) and flagella (Kim *et al*., 2007) (Fig. 2). *tofI* encodes an AHL synthase that produces HHL and OHL, whereas *tofR* encodes a QS signal receptor that binds to OHL (Kim *et al*., 2004, 2007) (Fig. 2). In the known regulatory cascade for the production and transport of toxoflavin, the main phytotoxin utilized by *B. glumae* as a major virulence factor, the TofR–OHL complex activates the expression of *toxJ* which encodes the transcriptional activator ToxJ; ToxJ induces the expression of *toxR* which encodes the LysR family regulatory protein ToxR (Kim *et al*., 2004) (Fig. 2). Finally, in the presence of the co-inducer toxoflavin, ToxR activates the expression of the *toxABCDE* and *toxFGHI* operons, which are responsible for the biosynthesis and transport of toxoflavin, respectively (Kim *et al*., 2004, 2009) (Fig. 2). ToxJ is also required for the expression of *toxABCDE* and *toxFGHI* operons, and TofR–OHL positively controls the expression of *tofI*, forming a positive autoregulatory feedback loop (Kim *et al*., 2004, 2009). The TofI/TofR QS system is also a central regulatory element of the known regulatory system for flagellar biogenesis and, consequently, flagellum-mediated swimming and swarming motilities of *B. glumae* (Kim *et al*., 2007) (Fig. 2). In this regulatory system, the TofR–OHL complex renders the expression of *qsmR* which encodes the IclR family regulatory protein QsmR; QsmR expresses the *flhDC* genes, encoding the regulatory protein complex FlhDC. FlhDC is, in turn, essential for the expression of flagellar biogenesis genes (Kim *et al*., 2007) (Fig. 2). Although it has been demonstrated that another major virulence gene, *lipA*, which encodes a lipase, is also dependent on the TofI/TofR QS system for its expression (Devescovi *et al*., 2007), intermediate regulatory factors that connect the QS system and the expression of *lipA* have not yet been reported (Fig. 2).

Meanwhile, an open reading frame (ORF) divergently transcribed from *tofR* and present in the intergenic region of *tofI* and *tofR* has been found to encode an RsaM homologue and to be highly conserved among *Burkholderia* spp. (Chen *et al*., 2012). Recently, *rsaM* has been discovered as a novel negative regulator of AHL-mediated QS systems in another rice pathogenic bacterium, *Pseudomonas fuscovaginae* (Mattiuzzo *et al*., 2011). *rsaM* of *Pseudomonas fuscovaginae* is also present in the intergenic region of the *luxI* and *luxR* homologues, *pfsI* and *pfsR*, respectively, and is divergently transcribed from *pfsR*, suggesting that the ORF encod-

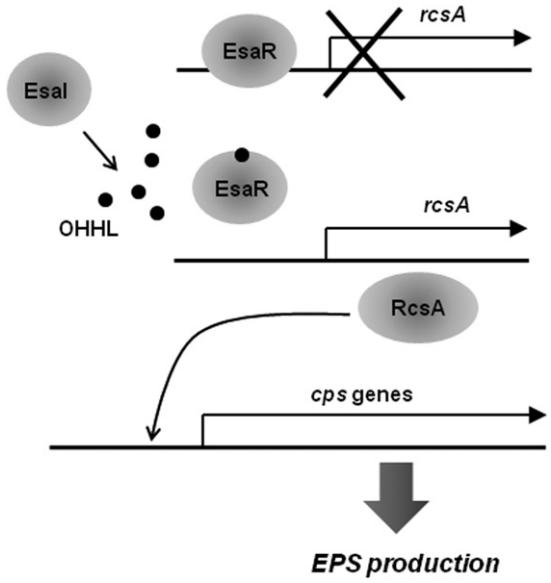

Fig. 3 Schematic view of the regulatory mechanism for the production of extracellular polysaccharide (EPS) in *Pantoea stewartii* ssp. *stewartii*. OHHL, *N*-3-oxohexanoyl-L-homoserine lactone.

ing a RsaM homologue in *B. glumae* may also have a regulatory function for the TofI/TofR QS system. Indeed, modulation of QS-dependent toxoflavin production by this ORF has been observed recently in preliminary experiments (Chen *et al*., 2012). The elucidation of the function of this new regulatory factor would substantially expand our knowledge of the QS systems of *Burkholderia* spp.

EsaI/EsaR of Pantoea stewartii *ssp.* stewartii

The QS system present in the corn pathogen *P. stewartii* ssp. *stewartii* is unusual when compared with the majority of AHL-mediated QS systems in at least two aspects. First, the EsaR AHL receptor negatively regulates the production of extracellular polysaccharides (EPS), important virulence factors of *P. stewartii* ssp. *stewartii*, in the absence of the cognate AHL compound *N*-3-oxohexanoyl-L-homoserine lactone (OHHL) (von Bodman *et al*., 1998) (Fig. 3). EsaR exerts this negative regulatory function through direct repression of the transcription of *rcsA*, which encodes an essential transcriptional activator for the expression of *cps* genes involved in the production of EPS (Minogue *et al*., 2005) (Fig. 3). Second, the cognate AHL produced by the EsaI AHL synthase, OHHL, antagonizes this repressive function of EsaR, resulting in the derepression of EPS production (von Bodman *et al*., 1998) (Fig. 3). *esaR* mutants producing excessive amounts of EPS show reduced virulence, poor adhesion to the surface and less compact biofilm structures, suggesting that the repression of EPS production by EsaR at the early stages of infection is critical for normal disease development caused by *P. stewartii* ssp. *stewartii* (Koutsoudis *et al*., 2006; von Bodman *et al*., 1998).

Similar to EsaR, several AHL receptors, including ExpR proteins of *Pectobacterium carotovorum* and *Pectobacterium chrysanthemi*, have also been shown to have repressive activities on the expression of virulence genes or virulence-related phenotypes in the absence of cognate AHL molecules, and their regulatory activities are neutralized by the addition of cognate AHL molecules (Andersson *et al*., 2000; Cui *et al*., 2005, 2006; Sjoblom *et al*., 2006).

QS systems mediated by DSFs

A DSF-mediated QS system was first discovered in the plant pathogenic bacterium *Xanthomonas campestris* pv. *campestris* (Tang *et al*., 1991), and later found to be widely distributed among plant pathogenic *Xanthomonas* spp. and *Xy. fastidiosa*, as well as other bacteria, including the *B. cepacia* complex, *Pseudomonas aeruginosa* and *Stenotrophomonas maltophilia* (Deng *et al*., 2011). The DSF of *X. campestris* pv. *campestris* was identified as an unsaturated fatty acid, *cis*-11-methyl-2-dodecenoic acid (Wang *et al*., 2004), whereas that of *Xy. fastidiosa* was identified as a saturated fatty acid, 12-methyl-tetradecanoic acid (Colnaghi Simionato *et al*., 2007). Additional structural variants of DSF molecules have also been identified from various Gram-negative bacteria (Deng *et al*., 2011). The biosynthesis of DSF is dependent on the *rpfF* gene, which encodes a putative enoyl-CoA hydratase in *Xanthomonas* spp. and *Xy. fastidiosa* (Barber *et al*., 1997; Newman *et al*., 2004). *rpfB*, which encodes a putative long-chain fatty acyl CoA ligase, is also involved in DSF biosynthesis in both *X. campestris* pv. *campestris* and *Xy. fastidiosa* (Almeida *et al*., 2012; Barber *et al*., 1997). A recent study by Bi *et al*. (2012) on Bcam0581, an RpfF homologue in the opportunistic human pathogen *B. cenocepacia*, provided an important clue for the functional mechanism of RpfF in the biosynthesis of DSF molecules. In that study, Bcam0581 was shown to have dual enzymatic activity: (i) dehydratase activity for the dehydration of 3-hydroxydodecanoyl-acyl carrier protein (ACP) to *cis*-2-dodecenoyl-ACP; and (ii) thioesterase activity for the cleavage of a thiolester bond to liberate the free fatty acid and produce BDSF, the DSF molecule of *B. cenocepacia* (Bi *et al*., 2012). DSF signal perception and transduction into target molecules in the cell are conferred by *rpfC* and *rpfG* genes, which encode a hybrid sensor kinase and a response regulator, respectively, comprising the RpfC/RpfG two-component regulatory system (Fig. 4). These *rpf* genes are clustered and conserved in a wide range of bacteria that possess a DSF-mediated QS system (He and Zhang, 2008).

The structural features of individual *rpf* gene products have been elucidated by several recent studies. Structural analyses of the crystal structure and sequence alignment of RpfF revealed a predicted substrate-binding pocket composed of hydrophobic residues (G85, L136, G137, G138, M170, W258 and L276) and two putative catalytic residues (E141 and E161) within the RpfF molecule (Cheng

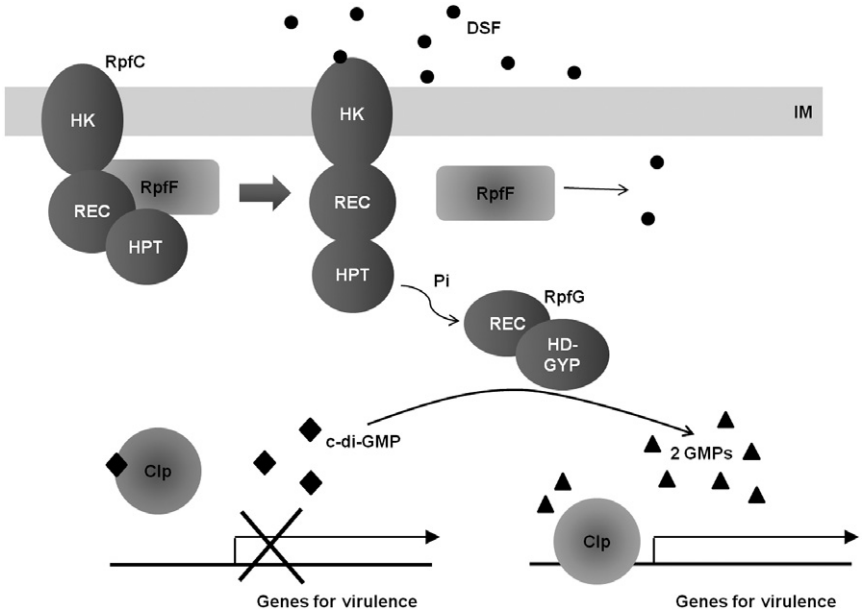

Fig. 4 Schematic model of the regulatory mechanism involving DSF and c-di-GMP for the virulence of *Xanthomonas campestris* pv. *campestris*. c-di-GMP, cyclic diguanosine monophosphate; DSF, diffusible signal factor; GMP, guanosine monophosphate; HK, histidine kinase domain; HPT, histidine transfer domain; IM, inner membrane; Pi, phosphate group; REC, receiver domain.

et al., 2010). Point mutations of these core residues resulted in the abrogation of enzymatic activity for DSF biosynthesis, indicating the importance of these structural elements for RpfF function (Cheng *et al*., 2010). RpfC is a histidine sensor kinase containing several functionally discrete domains, including a histidine kinase (HK) domain, five transmembrane (TM) domains, a receiver (REC) domain and a histidine transfer (HPT) domain (Slater *et al*., 2000) (Fig. 4). The phosphorelay is seemingly an essential part of RpfC function, because substitution of the conserved histidine residues in the HK and HPT domains or the conserved aspartate residue in the REC domain, which are predicted to be involved in the phosphorelay, abrogated the RpfC function in DSF-mediated QS (He *et al*., 2006). Intriguingly, RpfC also plays a negative regulatory role in DSF biosynthesis without much change in the transcription of *rpf* genes (Slater *et al*., 2000; Wang *et al*., 2004). He *et al*. (2006) found that RpfC controls DSF production at a post-translational level via direct physical interaction with RpfF, suggesting the following working model: in the absence (or at a lower than threshold level) of DSF, both RpfC and RpfF are in an inactive state because of their physical binding to each other; in the presence (or at a higher than threshold level) of DSF, RpfC changes its conformation on reception of the DSF signal and consequently releases RpfF, which activates both proteins for their signal production and transduction functions (Deng *et al*., 2011) (Fig. 4). This mode of action allows a positive feedback of RpfF activity without a substantial increase in *rpfF* transcription (He *et al*., 2006).

RpfG is a response regulator that contains a typical REC domain as well as an HD-GYP domain with phosphodiesterase (PDE) activity (Slater *et al*., 2000) (Fig. 4). Functional analyses of the RpfG protein indicated that both the REC and HD-GYP domains are essential for DSF signal transduction in *X. campestris* pv. *campestris* (Deng *et al*., 2011). In particular, it has been shown that RpfG functions as a juncture that connects the QS signalling mediated by DSF and the intracellular signalling mediated by c-di-GMP (Deng *et al*., 2011). The function of RpfG in the c-di-GMP signalling system is addressed in detail in the section on Intracellular signalling systems mediated by second messenger molecules.

rpf/DSF QS systems play substantially different roles in *Xanthomonas* spp. and *Xy. fastidiosa* even though the *rpf* gene cluster, which contains the *rpfG*, *rpfC* and *rpfF* genes, is conserved in both genera. Basically, the *rpf*/DSF QS system plays a positive role for the virulence of *Xanthomonas* spp., whereas it exerts a negative role in the virulence of *Xy. fastidiosa*. In *X. campestris* pv. *campestris*, the disruption of any gene in the *rpf* gene cluster caused reduced biosynthesis of extracellular enzymes and EPS, and, consequently, reduced virulence (Barber *et al*., 1997; Slater *et al*., 2000; Tang *et al*., 1991). In *Xy. fastidiosa*, however, *rpfF* mutants, which were unable to synthesize DSF signal molecules, were more virulent than the wild-type strain even though they were defective in vector transmission and biofilm formation in the insect vector (Newman *et al*., 2004). *rpfF* mutants also showed enhanced movement and multiplication in the xylem vessels relative to the wild-type, suggesting that the DSF-mediated QS system of *Xy. fastidiosa* was adapted to restrain bacterial virulence activities within host plants for the endophytic lifestyle of this bacterium (Chatterjee *et al*., 2008a). In contrast, *rpfC* mutants of *Xy. fastidiosa*, which overproduce DSFs and hyperexpress *rpfF*, showed reduced virulence, an inability to migrate in xylem vessels and increased expression of genes for the adhesion proteins FimA, HxfA and HxfB (Chatterjee *et al*., 2008b). Nevertheless, *rpfC* mutants of *Xy. fastidiosa* also showed impaired vector transmission similar to *rpfF* mutants (Chatterjee *et al*., 2008b). These differential regulatory functions of the

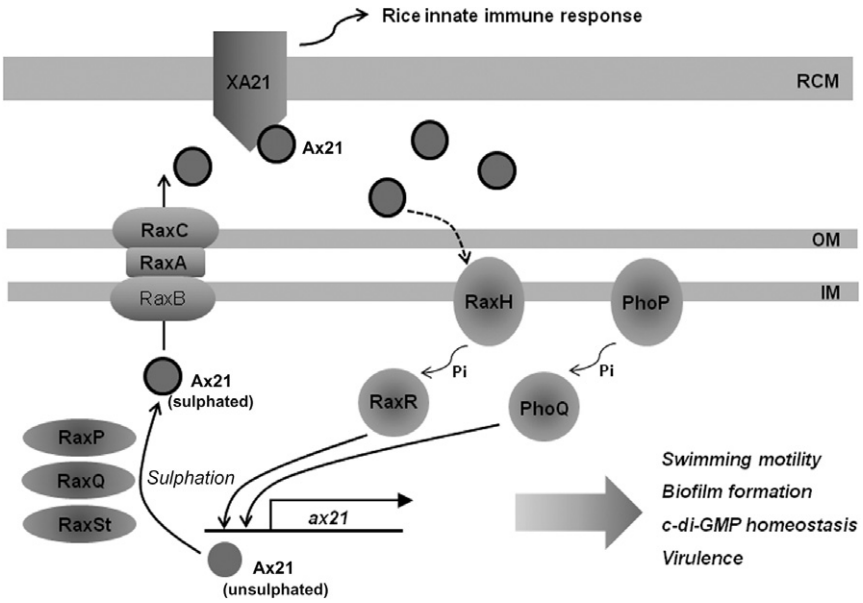

Fig. 5 Schematic model of Ax21-mediated quorum sensing and rice defence induction by *Xanthomonas oryzae* pv. *oryzae*. c-di-GMP, cyclic diguanosine monophosphate; IM, inner membrane; OM, outer membrane; Pi, phosphate group; RCM, rice cell membrane.

rpf/DSF QS system in virulence between *Xanthomonas* spp. and *Xy. fastidiosa* suggest that this signalling system functions differently in different pathogens depending on the pathogenic behaviour and lifestyle of the pathogen.

rpf/DSF QS systems were also studied in other plant pathogenic *Xanthomonas* spp., including the bacterial citrus canker pathogen, *X. axonopodis* pv. *citri* (Andrade *et al*., 2006), the bacterial rice leaf blight pathogen, *X. oryzae* pv. *oryzae* (Chatterjee and Sonti, 2002; He *et al*., 2010; Tang *et al*., 1996), the bacterial rice leaf streak pathogen, *X. oryzae* pv. *oryzicola* (Zhao *et al*., 2011), and the bacterial soybean pustule pathogen, *X. axonopodis* pv. *glycines* (Thowthampitak *et al*., 2008). Even though positive roles of the *rpf*/DSF QS system in virulence were well conserved among all of the *Xanthomonas* spp. studied, species- and strain-specific functional variations of the *dpf*/DSF QS system were observed within the genus. For example, extracellular enzyme production patterns of *rpfF* mutants were contradictory between two *X. oryzae* pv. *oryzae* strains from different geographical locations (Chatterjee and Sonti, 2002; He *et al*., 2010; Rai *et al*., 2012). *rpfF* was shown to positively regulate extracellular enzyme production in a strain of *X. oryzae* pv. *oryzae* from Korea (He *et al*., 2010), but to negatively regulate extracellular enzyme production in a strain from India (Chatterjee and Sonti, 2002; Rai *et al*., 2012). In addition, extracellular enzyme production was positively regulated by *rpfC* in *X. campestris* pv. *campestris*, but was not affected by *rpfC* in *X. oryzae* pv. *oryzae* (Slater *et al*., 2000; Tang *et al*., 1996).

QS systems mediated by peptide-type signal molecules (Ax21 family small proteins)

Recent seminal works by Pamela Ronald's research group have elucidated the function of a small bacterial protein, Ax21, as a QS signal in the rice pathogenic bacterium *X. oryzae* pv. *oryzae* (Han *et al*., 2011b, 2011a). This is the first example of signalling by peptide molecules in Gram-negative bacteria and provides great insight into the signalling networks of bacterial pathogens involving multiple signal molecules and pathways.

Ax21 was first identified as the ligand of XA21, a receptor kinase (or pattern recognition receptor, PRR) of rice responsible for the rice disease resistance derived from the gene-for-gene interaction between the resistance gene *Xa21* and the avirulence gene *avrXa21* (Fig. 5) (Lee *et al*., 2009). The rice resistance gene *Xa21* was first identified via fine genetic mapping (Song *et al*., 1995). The product of this resistance gene, XA21, is a membrane-spanning protein composed of the extracellular sensor domain and the cytoplasmic serine/threonine kinase domain (Song *et al*., 1995). The avirulence protein Ax21, which corresponds to XA21, was identified thorough serial purifications and accompanying tests for the avirulence activity of the extracellular fraction of the bacterial cell culture using reverse-phase high-performance liquid chromatography and nuclear magnetic resonance (Lee *et al*., 2009). Ax21 is a 194-amino-acid protein secreted through a type I secretion system, and its orthologues are present in plant and animal pathogens, including *Xanthomonas* spp. (90%–98% identity), *Xy. fastidiosa* (48% identity) and *Stenotrophomonas maltophilia* (61% identity).

Intriguingly, Ax21 contains two predicted tyrosine (Tyr) sulphation sites and can be reduced to the 17-amino-acid functional peptide that includes sulphated Tyr-22 (AxY^S22) (Lee *et al*., 2009). Sulphation of Tyr-22 is essential for the function of AxY^S22 for Ax21 activity, and the sulphated form of this peptide binds to XA21 with high affinity (Lee *et al*., 2009). It is also noteworthy that the amino acid sequence of AxY^S22 is highly conserved in the Ax21 family proteins of all the sequenced *Xanthomonas* spp. (100%

identity), *Xy. fastidiosa* (77% identity) and *S. maltophilia* (65% identity) (Lee *et al*., 2009). Probably as a result of this high level of similarity, Ax21 orthologues may be functionally interchangeable among *Xanthomonas* spp. (Lee *et al*., 2009; Ronald, 2011) and other related genera (McCarthy *et al*., 2011). Genes encoding Ax21 are conserved in all of the sequenced species of the genus *Xanthomonas* and other related genera, suggesting that this protein exerts an important function for the ecological or parasitic fitness of the bacteria in nature, including hosts without XA21 or equivalent host defence components (Ronald, 2011). In this regard, Ax21 can be considered as a pathogen-associated molecular pattern (PAMP) (Bent and Mackey, 2007), and the XA21-mediated rice innate immune pathway may be a promising target to promote stable rice disease resistance against *X. oryzae* pv. *oryzae*.

Genetic studies of *X. oryzae* pv. *oryzae* have revealed that three groups of bacterial genes are required for the function of Ax21 in the activation of the XA21-dependent rice disease resistance: (i) three genes for a type I secretion system (*raxA*, *raxB* and *raxC*); (ii) three genes for the sulphation of Ax21 (*raxP*, *raxQ* and *raxSt*); and (iii) four genes for two two-component regulatory systems (*raxR*, *raxH*, *phoP* and *phoQ*) (Fig. 5) (Ronald, 2011). Recently, Han *et al*. (2011b) found that the expression of these *rax* (required for Ax21 activity) genes is dependent on bacterial density, and that Ax21 functions as the signal molecule for this QS. In the same study, a Δ*ax21* derivative of the wild-type strain, PXO99, showed reduced biofilm formation, swimming motility and bacterial growth in rice leaves compared with the wild-type, suggesting that Ax21-mediated QS also controls these phenotypic traits of *X. oryzae* pv. *oryzae* (Fig. 5) (Han *et al*., 2011b). In particular, the Δ*ax21* strain, PXO99Δ*ax21*, showed reduced ability to cause symptoms and grow in rice leaves at a low cell density, which may mimic natural inoculation conditions, suggesting that Ax21-mediated QS is critical to the initiation of infection at a low level of inoculum in nature. Microarray data presented by the same study supported the observed phenotypes of PXO99Δ*ax21*, in which genes involved in bacterial motility (including *fliC*, *fliD* and *pilG*), biofilm formation (including *gumJ*, *gumE* and *gumK*) and c-di-GMP metabolism (nine genes containing putative HD-GYP, EAL and GGDEF domains) were up-regulated in the wild-type relative to the Δ*ax21* strain (Han *et al*., 2011b; Ronald, 2011). The expression of the *rpf* operon was not affected significantly by Ax21, suggesting that the Ax21-mediated QS system has little overlap with the DSF-mediated QS system, and that each system plays an independent role in density-dependent gene expression in *X. oryzae* pv. *oryzae* (Ronald, 2011).

A unique feature of Ax21 is that this protein exerts dual functions: as a PAMP triggering a rice innate immune system through direct binding to a PRR, and as a QS signal molecule controlling bacterial genes (Han *et al*., 2011b; Lee *et al*., 2009). Even though the Ax21-mediated QS system has been studied mainly with *X. oryzae* pv. *oryzae* and rarely with other bacteria, the presence of *ax21* and *rax* gene orthologues in other *Xanthomonas* spp. and closely related genera strongly suggests that this novel QS system is a common signalling system in at least certain groups of bacterial pathogens, especially those belonging to *Xanthomonas* spp. and *Xy. fastidiosa*.

QS systems mediated by other types of signalling molecule

AI-2 molecules are synthesized by *S*-ribosylhomocysteinase (LuxS) encoded by *luxS* genes (Pereira *et al*., 2012; Roy *et al*., 2011). Specifically, LuxS catalyses the conversion of *S*-ribosylhomocysteine (SRH) to 4,5-dihydroxy-2,3-pentanedione (DPD). In turn, DPD undergoes intramolecular cyclization, forming different isoforms, which are collectively referred to as AI-2 (Pereira *et al*., 2012; Roy *et al*., 2011). In *V. harveyi*, where AI-2 was first found, the AI-2 molecule is a borated form of *S*-2-methyl-2,3,3,4-tetrahydroxytetrahydrofuran (*S*-THMF), *S*-THMF-borate (Chen *et al*., 2002). A markedly different isomer of DPD without boron, *R*-2-methyl-2,3,3,4-tetrahydroxytetrahydrofuran (*R*-THMF), was found to be the AI-2 molecule in *Salmonella enterica* ssp. *enterica* serovar Typhimurium (Miller *et al*., 2004). The production of AI-2 molecules is widespread among various species of Gram-positive and Gram-negative bacteria, and these signal molecules are considered to be a 'universal language' for interspecies' communication in the bacterial community (Bassler and Losick, 2006; Lowery *et al*., 2008; Pereira *et al*., 2012; Sun *et al*., 2004). Since AI-2 molecules were first reported to function as a second QS signal for bioluminescence in *V. harveyi* (Bassler *et al*., 1994) and for virulence and biofilm formation in *Vibrio cholerae* (Miller *et al*., 2002), numerous studies have demonstrated diverse functions of AI-2 molecules, including biofilm formation, motility and virulence factor production, in various bacterial species (Pereira *et al*., 2012).

In spite of the recent advancement in AI-2 studies, the signalling function of *luxS*/AI-2 in plant pathogenic bacteria has not been widely studied and is somewhat ambiguous. In *Pe. carotovorum* ssp. *carotovorum* and *Pe. carotovorum* ssp. *atrosepticum*, the production of extracellular enzymes was reduced in *luxS* mutants when compared with the wild-type parental strains (Coulthurst *et al*., 2006). In *Erwinia amylovora*, QS mediated by LuxS was not observed and inactivation of *luxS* did not cause any significant change in virulence-related phenotypes, other than reduced expression of the virulence genes, *hrpL* and *dspA/E* (Rezzonico and Duffy, 2007). However, another study by Gao *et al*. (2009) with the same strain of *E. amylovora* showed that mutation of *luxS* caused impaired motility, tolerance to reactive oxygen species and virulence. Despite the different conclusions made by these research groups on the role of LuxS in the virulence of *E. amylovora*, it should be noted that genes encoding known AI-2 receptors

required for AI-2 QS, LuxP or LsrB, could not be found in *E. amylovora* (Rezzonico and Duffy, 2007) or in other plant pathogenic bacteria (Rezzonico and Duffy, 2008). This suggests that any effect caused by a *luxS* mutation may be a result of impaired metabolic function rather than signalling function (Rezzonico and Duffy, 2008). Nevertheless, it cannot be ruled out that unknown, alternative AI receptors might exist for AI-2 QS signalling in some plant pathogenic bacteria.

QS systems mediated by oligopeptides have been investigated in several Gram-positive bacteria, including *Staphylococcus aureus*, *Bacillus subtilis*, *Enteroccocus faecalis* and the *Bacillus cereus* group (Antunes *et al*., 2010; Rocha-Estrada *et al*., 2010; Shank and Kolter, 2011). However, not much is known about this type of QS signal molecule in Gram-positive plant pathogenic bacteria. In addition, 2-heptyl-3-hydroxy-4-quinolone (*Pseudomonas* quinolone signal, PQS) and its immediate precursor, 2-heptyl-4-quinolone (HHQ), are known to act as QS signal molecules to control global gene expression for many virulence factors and quinolone biosynthesis in the opportunistic human pathogen *Ps. aeruginosa* (Antunes *et al*., 2010). However, as with the oligopeptides, the signalling functions of PQS and HHQ in plant pathogenic bacteria remain unknown.

INTRACELLULAR SIGNALLING SYSTEMS MEDIATED BY SECOND MESSENGER MOLECULES

Signalling systems mediated by c-di-GMP

c-di-GMP is a small diffusible signal molecule that influences a wide range of cellular processes, including flagellum-mediated motility, cell cycle and EPS biosynthesis, as well as bacterial virulence (Romling, 2012). c-di-GMP signalling systems are generally composed of three major components: diguanylate cyclases (DGCs), PDEs and c-di-GMP-binding effectors (Mills *et al*., 2011; Sondermann *et al*., 2012). DGCs synthesize c-di-GMP from two guanosine triphosphate (GTP) molecules, whereas PDEs degrade c-di-GMP, generating a single pGpG molecule or two GMP molecules (Sondermann *et al*., 2012). In general, the GGDEF domain of DGCs and the EAL or HD-GYP domains of PDEs are responsible for DGC and PDE activities, respectively, and balanced control of these opposite activities of DGCs and PDEs determines c-di-GMP homeostasis within the cell (Sondermann *et al*., 2012). DGC and PDE activities are modulated through various mechanisms, including protein–protein interaction and phosphorylation, in accordance with environmental cues and cell–cell communication. Thus, cellular levels of c-di-GMP are an integral output of bacterial sensory systems that perceive various biotic and abiotic conditions. Finally, c-di-GMP translates input signals into the modulation of cellular behaviour by binding to diverse downstream effector molecules, including specific c-di-GMP receptor proteins with a characteristic 'PilZ' domain, degenerate GGDEF/EAL domains and c-di-GMP-specific transcriptional factors (i.e. Clp) and RNA motifs (riboswitches) (Mills *et al*., 2011; Sondermann *et al*., 2012).

Although an early study revealed the regulatory function of c-di-GMP on cellulose production by the plant pathogen *A. tumefaciens* (Amikam and Benziman, 1989), not much was known about the c-di-GMP signalling system in plant pathogenic bacteria until recently. Recent seminal studies on *X. campestris* pv. *campestris* and *X. axonopodis* pv. *citri* have provided great insights into the function of c-di-GMP in virulence, in conjunction with the QS mediated by DSF molecules and the RpfC/RpfG two-component regulatory system (Ryan and Dow, 2010; Zhang, 2010). Ryan *et al*. (2006) demonstrated with *X. campestris* pv. *campestris* that RpfG containing the HD-GYP domain degraded c-di-GMP, and that this PDE activity was dependent on the conserved H and D residues of the HD-GYP domain. In the same study, the conserved H and D residues were also required for the regulatory activity of RpfG in the biosynthesis of virulence factors (Ryan *et al*., 2006). This regulatory function associated with the key residues for PDE activity is probably exerted through the c-di-GMP-binding protein, Clp (Tao *et al*., 2010). Clp, a Crp [cyclic adenosine monophosphate (cAMP) receptor protein] family protein, is an essential component of the *rpf*/DSF-mediated QS system that regulates the expression of virulence genes of *X. campestris* pv. *campestris*, including the genes for flagellar biogenesis, the Hrp type III secretion system, extracellular enzymes and EPS synthesis (He *et al*., 2007). Unlike most Crp family proteins, Clp has a high level of binding affinity to its target DNA in the absence of any ligand, and c-di-GMP allosterically inhibits the DNA-binding activity of Clp (Chin *et al*., 2010; Leduc and Roberts, 2009). This inhibitory action of c-di-GMP on Clp is probably a result of the direct binding of c-di-GMP to the Clp protein, because mutations of the Clp residues predicted to be involved in c-di-GMP binding through structural modelling processes caused substantial reductions in the binding affinity of Clp to c-di-GMP, the inhibitory effect of c-di-GMP on DNA binding of Clp and virulence (Chin *et al*., 2010; Tao *et al*., 2010).

However, it was also revealed that RpfG exerted a subset of its functions, including motility, through physical interactions with DDGEF domain proteins in *X. campestris* pv. *campestris* (Ryan *et al*., 2010). Similarly, the RpfG protein of *X. axonopodis* pv. *citri* was observed to interact with GGDEF domain proteins in yeast two-hybrid (YTH) experiments (Andrade *et al*., 2006). The interaction of RpfG with DDGEF domain proteins and the RpfG functions associated with the protein–protein interaction were dependent on the G, Y and P residues of the HD-GYP domain, but not on the H and D residues and their associated PDE activity (Ryan *et al*., 2010). In the same study, two GGDEF domain proteins (XC0249 and XC0420) were confirmed through YTH and fluorescence resonance energy transfer (FRET) assays to be RpfG-interacting proteins; in addition, simultaneous mutation of the two genes

encoding the RpfG interactors caused the loss of pilus-dependent motility and reduced virulence, as observed with alanine substitution of the G, Y or P residue in the HD-GYP domain of the RpfG protein (Ryan *et al*., 2010).

Meanwhile, McCarthy *et al*. (2008) reported that *X. campestris* pv. *campestris* contains at least four genes that produce proteins with a PilZ or c-di-GMP-binding domain. In animal pathogens, PilZ domain proteins play important roles in c-di-GMP signalling for the control of specific cellular processes, including virulence (Cotter and Stibitz, 2007; Schirmer and Jenal, 2009). Among the four genes that encode proteins with a PilZ domain (*XC0965*, *XC2249*, *XC2317* and *XC3221*) in *X. campestris* pv. *campestris*, mutation of *XC0965* and *XC2249* caused reduced production of extracellular enzymes, whereas mutation of *XC2249* and *XC3221* impaired motility (McCarthy *et al*., 2008). It was also demonstrated that XC6012, a PilZ domain protein that is identical to XC2249, but from another strain of *X. campestris* pv. *campestris*, was important for virulence and formed a unique tetrameric structure (Li *et al*., 2011).

A recent study by Ryan *et al*. (2012) has illustrated how the physical interaction of RpfG with GGEDF domain proteins controls pilus-dependent motility through the PilZ domain protein XC2249. In this study, RpfG-GGDEF domain protein complexes recruit the PilZ domain protein XC2249 as an adaptor for further interaction with the pilus motor proteins PilU and PilT (Ryan *et al*., 2012).

On the basis of these studies, the c-di-GMP signalling function mediated by RpfG may consist of two levels. The first level of RpfG function is dependent on the PDE activity of the HD-GYP domain (H and D are key residues) and controls bacterial motility and extracellular enzyme biosynthesis (Ryan *et al*., 2006, 2010). This signalling pathway may function through the following mechanism: the HD-GYP domain of RpfG degrades c-di-GMP, resulting in decreased c-di-GMP levels in bacterial cells; Clp is then liberated from its inhibitory ligand, c-di-GMP, to promote the expression of virulence genes (Fig. 4). The second level of RpfG function is dependent on the physical interaction of the HD-GYP domain with GGDEF domain proteins (G, Y and P are key residues) and is responsible for only a subset of the functions of RpfG, such as bacterial motility (Ryan *et al*., 2010, 2012). This signalling function of RpfG for motility involves direct physical interactions with the motor proteins PilU and PilT, using the PilZ domain protein XC2249 as a juncture (Ryan *et al*., 2012).

Several studies have also revealed additional c-di-GMP signalling systems in plant pathogenic bacteria. In *X. campestris* pv. *campestris*, another two-component regulatory system, RavS/RavR, has been shown to regulate bacterial virulence factors through Clp, like the Rpf/DSF QS system (He *et al*., 2009). In this system, RavR, like RpfG, contains an EAL domain that shows PDE activity, whereas RavS includes two PAS domains implicated in the sensing of low-oxygen conditions; these domains have been shown to be essential for the regulatory functions of RavS and RavR in the production of virulence factors (EPS and extracellular enzymes), transcription of *clp* and bacterial virulence in host plants (He *et al*., 2009). Additive roles of the RavS/RavR and *rpf*/DSF systems in the production of virulence factors, *clp* expression and virulence have also been observed from single and double mutants of *X. campestris* pv. *campestris* for these systems, suggesting that Clp, a global regulator for *X. campestris* pv. *campestris* virulence, is modulated by the two separate signalling pathways that sense population density and hypoxia (He *et al*., 2009). In *Pe. carotovorum* ssp. *atrocepticum*, it has been found recently that the intracellular level of c-di-GMP affects the secretion of a multi-repeat adhesion protein (MRP), motility, biofilm formation and virulence (Perez-Mendoza *et al*., 2011a, 2011b), and that two novel genes encoding a putative PDE and a putative DGC modulate the intracellular c-di-GMP level in this plant pathogen (Perez-Mendoza *et al*., 2011a). A genetic study by Perez-Mendoza *et al*. (2011a) revealed that the putative PDE gene, *ECA3271*, suppressed the accumulation of secreted MRP and increased swimming motility via depletion of intracellular c-di-GMP, whereas the putative DGC gene, *ECA3270*, accounted for opposite phenotypes via elevation of the c-di-GMP level in the cell. Nevertheless, mutation of either *ECA3270* or *ECA3271* caused a significant reduction in bacterial virulence, suggesting that fine tuning of the cellular c-di-GMP level via balanced actions of DGC and PDE is crucial for host infection (Perez-Mendoza *et al*., 2011a). In another soft rot-causing plant pathogen, *Dickeya dadantii* (syn. *Pe. chrysanthemi*), two proteins containing an EAL domain (putative PDEs) were found to control multiple cellular behaviours, including biofilm formation and motility, and virulence gene expression (Yi *et al*., 2010). Both *ecpB* and *ecpC* were revealed to play a positive role in swarming/swimming motility, pectate lyase production and gene expression for the type III secretion system and type III effectors, but a negative role in biofilm formation (Yi *et al*., 2010). In the same study, EcpC was shown to have PDE activity that hydrolyses c-di-GMP into linear pGpG, suggesting that these EAL domain proteins exert their regulatory functions through the modulation of c-di-GMP levels (Yi *et al*., 2010).

Signalling pathways mediated by other cyclic nucleotide molecules

cAMP has long been known to play an important role in various cellular processes from the catabolism of alternative sugars to motility and virulence (Gomelsky, 2011). In soft rot-causing plant pathogenic bacteria, the cAMP–CRP (cAMP receptor protein) complex has been reported to positively control the genes for pectolysis (Nasser *et al*., 1997; Reverchon *et al*., 1997; Thomson *et al*., 1999).

In recent studies, cyclic di-AMP (c-di-AMP) and cGMP have also been found to function as signalling molecules in some bacteria. In *B. subtilis*, c-di-AMP was reported to be an important messenger

controlling bacterial sporulation in a DNA integrity-dependent manner (Oppenheimer-Shaanan *et al*., 2011; Witte *et al*., 2008), and to play an essential role in peptidoglycan (cell wall) homeostasis (Luo and Helmann, 2012). In *Staphylococcus aureus*, c-di-AMP was found to play a role in controlling cell size and in coping with stresses on cell membranes and walls (Corrigan *et al*., 2011). c-di-AMP may also be essential for viability in many bacteria because genes encoding di-adenylate cyclase domains, which are responsible for the biosynthesis of c-di-AMP, are conserved in a wide range of bacterial genomes, and mutations of these genes were lethal in tested bacteria belonging to *Listeria*, *Streptococcus* and *Mycoplasma* (French *et al*., 2008; Glass *et al*., 2006; Song *et al*., 2005; Woodward *et al*., 2010). Little is known about the signalling functions of cGMP in prokaryotes, but it has been demonstrated recently that cyst formation of *Rhodospirillum centenum* is dependent on cGMP and that the guanylyl cyclase gene cluster involved in cGMP production is conserved in a number of cyst-forming bacteria, including *Sinorhizobium meliloti* (Marden *et al*., 2011). Even though little is known about the signalling functions of these additional cyclic nucleotide signal molecules in plant pathogenic bacteria, this is an interesting research subject to pursue to expand our knowledge of signalling systems in these bacteria.

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

Signalling systems involved in the regulation of virulence genes in plant pathogenic bacteria have been discussed in this review. Even though individual signalling systems have been discussed separately, a single bacterial pathogen often possesses multiple signalling systems that are functionally interconnected with each other and comprise a global signalling network. For example, the signalling systems for the expression of virulence genes in *Xanthomonas* spp. are integrated forms of the DSF-mediated QS and c-di-GMP signalling systems. Recent studies on RpfG functions involving interactions with GGDEF and PilZ domain proteins in *X. campestris* pv. *campestris* (Ryan *et al*., 2010, 2012) and on the involvement of DSF in strong interactions of these c-di-GMP signalling components indicate that the QS and c-di-GMP signalling systems constitute an integrated sensory/regulatory pathway in this pathogen (Ryan *et al*., 2010). Similarly, it has been observed that QS is linked to c-di-GMP for the control of swarming activity in the animal pathogen *Vibrio parahaemolyticus* (Trimble and McCarter, 2011).

AHL-mediated QS systems in diverse bacterial species, including numerous plant pathogenic bacteria, have been studied extensively in recent decades. Nevertheless, signalling and regulatory mechanisms associated with AHL molecules have yet to be fully understood. In particular, it is extremely challenging to characterize precisely the QS systems of some bacteria that possess multiple *luxI* and *luxR* homologues because of the complexity derived from interactions of more than one set of LuxI/LuxR family proteins. Unknown accessory components that function in QS systems may also add to the complexity of QS systems. Recently, negative regulatory genes of QS systems have been discovered in the intergenic regions of various sets of *luxI* and *luxR* homologues (Venturi *et al*., 2011). It is very probable that additional accessory components may play crucial roles in AHL-mediated QS systems, even though they still remain to be identified.

DSF-mediated QS systems connected with c-di-GMP signalling systems are essential for the cell–cell communication of important plant pathogenic bacteria belonging to *Xanthomonas* and *Xy. fastidiosa*. More information is needed to comprehensively understand this type of QS. The Ax21 system is a newly identified QS system, especially for *Xanthomonas* spp. and *Xy. fastidiosa*. The functional relationship of this QS system with the known signalling and regulatory systems of *Xanthomonas* spp. and *Xy. fastidiosa* is an emerging research subject, and studies elucidating these relationships will provide a better understanding of the integrated signalling networks mediated by DSF, c-di-GMP and Ax21.

The control of plant diseases caused by bacterial pathogens is difficult because only a few antibiotics are permissible for use for agricultural purposes and because antibiotic-resistant strains are easily generated from the repeated use of antibiotics. The intercellular and intracellular signalling systems of bacterial pathogens are promising targets for new disease control strategies because they tend to be fundamentally different from those of eukaryotic organisms and they globally control multiple virulence factors and biofilm formation. A better understanding of bacterial signalling systems would provide important clues for the development of methods to inhibit signalling, which would result in the suppression of bacterial infection. Several approaches can be proposed for the inhibition of, or interference with, bacterial signalling systems including: (i) the development of synthetic or natural compounds that perturb the bacterial signalling systems; (ii) the employment of enzymes that degrade bacterial signal molecules; and (iii) the development of host plants that constitutively produce bacterial signal molecules to provide false signals to bacterial cells.

Recent exciting findings from the study of mammalian cells include the stimulation of innate immune systems on recognition of the bacterial signal molecules, c-di-GMP (Burdette *et al*., 2011) and c-di-AMP (Woodward *et al*., 2010). A TM protein of mammalian cells, STING (stimulator of interferon genes), was found to activate an innate immune pathway via the direct recognition of cyclic dinucleotides secreted by bacterial pathogens (Burdette *et al*., 2011). The interaction between XA21 in rice and Ax21 in *X. oryzae* pv. *oryzae* is an excellent example which illustrates that plants also possess similar innate immune systems that induce defence reactions through the recognition of bacterial signal molecules (Lee *et al*., 2009). The identification and characterization of the plant defence components that recognize other bacterial

signal molecules and subsequently activate defence responses are important research subjects for the study of plant–bacterial interactions, as well as for the development of new control strategies for bacterial plant diseases.

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