Role of Cyclic di-GMP in the Bacterial Virulence and Evasion of the Plant Immunity

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Abstract

Plant pathogenic bacteria are responsible for the loss of hundreds of millions of dollars each year, impacting a wide range of economically relevant agricultural crops. The plant immune system detects conserved bacterial molecules and deploys an arsenal of effective defence measures at different levels; however, during compatible interactions, some pathogenic bacteria suppress and manipulate the host immunity and colonize and infect the plant host. Different bacteria employ similar strategies to circumvent plant innate immunity, while other tactics are specific to certain bacterial species. Recent studies have highlighted the secondary messenger c-di-GMP as a key molecule in the transmission of environmental cues in an intracellular regulatory network that controls virulence traits in many plant pathogenic bacteria. In this review, we focus on the recent knowledge of the molecular basis of c-di-GMP signalling mechanisms that promote or prevent the evasion of bacterial phytopathogens from the plant immune system. This review will highlight the considerable diversity of mechanisms evolved in plant-associated bacteria to elude plant immunity.

An overview of bacterial plant diseases

Plants coexist with a myriad of microorganisms, bacteria, viruses or fungi, some of which deplete plant energy and resources. In particular, more than 100 bacterial species are responsible for many serious plant diseases worldwide, and many of these bacteria are responsible for important economic losses in agriculture, reducing the quantity and quality of produce. The bacteria *Xanthomonas oryzae* pv. *oryzae* is responsible for rice leaf blight, a disease that is estimated to cause a 50% economic loss in the annual rice yields worldwide (He *et al.*, 2010). The fire blight infection of apples, pears and other species from the

Rosaceae family results from the pathogen *Erwinia amylovora* and is estimated to cost €11.8 million in annual inspection in nurseries and garden centres in the EU (Caspari, 2011). The bacteria *Xylella fastidiosa* causes different diseases in many economically important crops, including Pierce's disease of grapevines, citrus variegated chlorosis and, more recently, olive quick decline syndrome (Purcell, 2013; Guan *et al.*, 2015). The Centre for Wine Economics predicted that the annual cost of Pierce's disease is US$61 million, and Pierce's disease control programmes have resulted in savings of US$261 million (Fuller *et al.*, 2012). Not surprisingly, *X. fastidiosa* was the first phytopathogen sequenced (Simpson *et al.*, 2016). *Xanthomonas arboricola* pv. pruni is the causal agent for the canker of many stone fruits, and the disease outbreak, affecting 30% of the produce, is estimated to cause economic losses of approximately €11.200 per ha (Stefani, 2010). The olive knot disease is one of the most serious diseases of olive trees, causing major damage on young trees and affecting the vigour, growth and total yield. It is caused by the pathogen *Pseudomonas savastanoi*, a bacterium belonging to the *Pseudomonas syringae* complex (Mansfield *et al.*, 2012). Although it is considered one of the most relevant diseases affecting the olive crop, economic losses as a result of the disease have not ben accurately estimated (Ramos *et al.*, 2012). This critical situation, considering the quality and quantity of the crop yield elicited by pathogens, is amplified with the increasing human population and the imperative need for an adequate food supply. A report by the Food and Agriculture Organization of the United Nations estimated a 50% increase in the global demand for food supply for 2030–2050 (World agriculture: towards 2030/2050, FAO, 2006).

The acknowledgement that microorganisms were ultimately a cause and not a consequence of plant diseases increased the efforts towards understanding the mechanisms behind plant diseases. In the early twentieth century, the first specific enzymes and toxins involved in the pathogenicity of plant pathogens were identified. In 1950, some plant pathogens that produce the plant hormone indolacetic acid (IAA), which is associated with bacterial pathogenicity, were identified. Since the 80s, the plant pathology field has benefited from the progress and development of molecular biology techniques. In particular, molecular tools have contributed to the detection, identification, isolation and modification of genes involved in disease resistance and pathogen virulence. Currently, the plant bacterial pathology field has enormously gained from the discovery of novel mechanisms that bacterial phytopathogens have evolved to interact with plants to successfully manipulate the host response and cause disease. Among these mechanisms, microbial pathogens produce diverse molecules, including nucleic acids, chitooligosaccharides and a great variety of effector proteins, that ultimately impact the host immune system (Pel and Pieterse, 2013; Le Fevre *et al.*, 2015).

The understanding of the plant defence machinery and the different strategies used by plant pathogens to overcome these barriers will contribute to the production of foods in the following two complementary ways: the protection of the food supply by combating pathogen infections and the development of highly disease-resistant plants in the near future.

Innate versus adaptive immunity in plants

Plants have evolved a great variety of mechanisms to protect themselves against pathogens, including the production of antimicrobial compounds, such as reactive oxygen species

(ROS), phytoalexins, chitinases, denfensins, oxylipins, and proteinases, and the induction of local and/or systemic acquired defence (Freeman, 2008). In contrast to mammals, plants lack an adaptive immune system and mobile defender cells, which enable the organism to have an extraordinary memory for future infections together with an infinite diversity of protection. However, plants and animals share some commonalities in the components of the innate immunity, such as the production of antimicrobial compounds and programmed cell death (Iriti and Faoro, 2007). Despite the absence of acquired or adaptive immunity, plants possess sophisticated and complex surveillance mechanisms to efficiently recognize and respond against putative pathogens. Plant immunity is based on the following two aspects: (i) a cell-autonomous basal resistance, also called innate immunity, determined by each individual cell (gene-for-gene response) and (ii) an unspecific acquired systemic resistance (SAR), where signals originating in the infection site spread to the uninfected parts of the plant. Given the localized response of plant innate immunity, compared with the animal immune system, the majority of plant cell types produce a broad arsenal of antimicrobial defences (Spoel and Dong, 2012).

The plant innate immunity has two different and consecutive levels (Jones and Dangl, 2006). The first more ancient and generalist level consistently remains ready to respond and involves cell surface proteins, called pattern recognition receptors (PRRs), which recognize specific microbial- or pathogen-associated molecular patterns (MAMPs or PAMPs), such as flagellin, peptidoglycan, elongation factor Tu and lipoteichoic acid among many others (Gust *et al.*, 2007; Pel and Pieterse, 2013; Newman *et al.*, 2013). At least two types of PRRs in plants are involved in bacterial MAMP recognition as follows: (i) the receptor-like kinases (RLKs) with an N-terminal extracellular domain, a single transmembrane region and a cytoplasmic kinase domain and (ii) the receptor-like proteins (RLPs), which differ from RLKs as they lack the intracellular kinase domain (Newman *et al.*, 2013). This recognition system is also present in animal innate immunity; however, plants have no clear Toll and Toll-like receptors, which is an important group of PRRs in animals (Hayashi *et al.*, 2001). Bacterial molecular patterns are generally immutable and conserved; however, the protein receptors involved in the recognition of similar molecular patterns are different in animals and plants. For example, the Toll-like receptor in mammals detects the bacterial flagellin, whereas the transmembrane receptor kinase FLS2 in plants directly interacts with flagellin (Gómez-Gómez and Boller, 2000; Smith *et al.*, 2003). The fact that PAMPs are highly conserved structures (essential in microbial recognition) and that their recognition varies depending on the organism suggests that microbial detection by different innate immunity systems has potentially converged (Nürnberger *et al.*, 2004; Zipfel and Felix, 2005). An immense list of immune receptors compensates for the lack of a pathogen-specific immune response in plants (Ausubel, 2005), particularly in *Arabidopsis thaliana*, which possesses more than 600 RLKs, whereas there are approximately 20 TLRs in mammals (Hopkins and Sriskandan, 2005; Ausubel, 2005; Willmann *et al.*, 2011).

Early pathogen perception elicits PAMP-triggered immunity (PTI), which is the first plant defence response and involves callose deposition and ROS production to restrict bacterial colonization and dispersal among the plant tissues (Trda *et al.*, 2015). However, some pathogens are able to mask this PTI response with the translocation of a large number of different effectors via the type III secretion system (T3SS), resulting in effector-triggered susceptibility (ETS) (Fig. 8.1) (Jones and Dangl, 2006; Win *et al.*, 2013; Castañeda-Ojeda

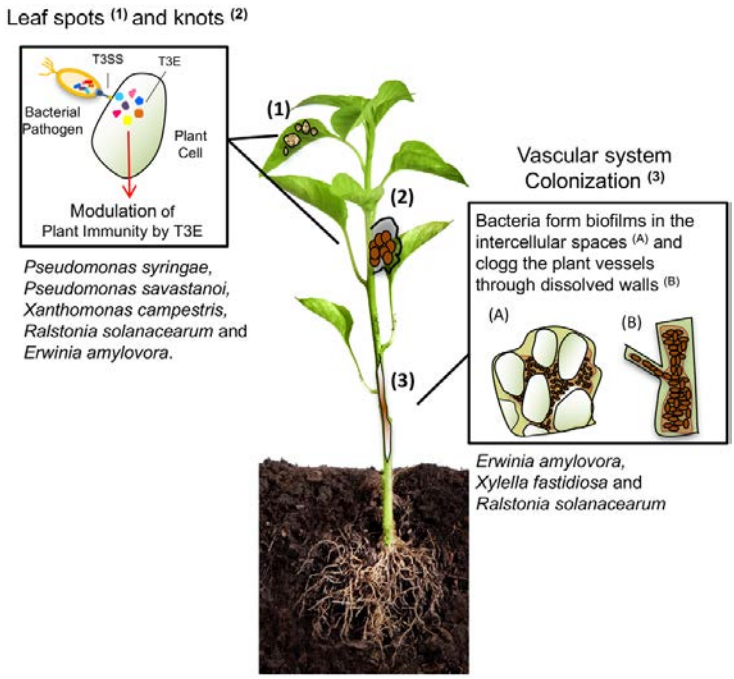

Figure 8.1 Virulence mechanisms in relevant plant pathogenic bacteria and the kinds of symptoms they cause. Bacteria as *Xanthomonas campestris*, *Ralstonia solanacearum*, *Erwinia amylovora* and bacteria belonging to the *Pseudomonas syringae* complex among others, cause leave spots and knots, primarily through the secretion of type III secretion system (T3SS) effectors (T3E) which interfere with the plant immune system, allowing further colonization of the plant tissues and survival within the plant. Some bacteria as *E. amylovora*, *R. solanacearum* and *Xylella fastidiosa* besides other virulence factors produce a variety of degradative enzymes and extracellular polysaccharides that will promote biofilm formation within the plant apoplast and further colonization of the plant vascular system protecting bacteria from the plant defences and causing disease.

et al., 2016; Washington *et al.*, 2016). Then, plants activate a second level of resistance that is mediated by the plant cytoplasmic NB-LRR receptors (encoded by *R genes*), which directly or indirectly recognize, bind and, therefore, block some of these bacterial effectors, causing effector-triggered immunity (ETI), a disease resistance condition that leads to a hypersensitive cell-death response (HR) during incompatible interactions (Dodds and Rathjen, 2010; Ishikawa *et al.*, 2014). However, this description has been simplified, as pathogens are constantly acquiring new strategies to overcome plant defences, and simultaneously plants co-evolve to recognize and respond to new bacterial equipment in a fascinating endless 'tug of war' game between plants, bacteria and natural selection.

Both pathogenic and commensal/beneficial bacteria on the plant surface are initially recognized as harmful organisms similar to animal innate immunity (Pel and Pieterse, 2013). Interestingly, the microbiome of plants and animals plays a key role in the adaptation of the host to its environment and contributes to the development of the immune system (Guttman *et al.*, 2014; Sun *et al.*, 2015). Consequently, some bacteria utilize different strategies to counterattack plant immunity at different levels. This strategy is well exemplified in

P. syringae or *Xanthomonas campestris*, two bacterial species that reduce the expression of flagellar genes or present polymorphisms of the flg22 epitope (Wenxian Sun *et al.*, 2006; Lan *et al.*, 2006; Wang *et al.*, 2015). In animals, important pathogens, such as *Helicobacter pylori* or *Campylobacter jejuni*, modify the entire flagellin recognition site to evade perception through the Toll-like receptor 5 (TLR5) (Purcell, 2013; Guan *et al.*, 2015; Broz and Monack, 2013). In general, plant pathogenic bacteria use different strategies to suppress plant defences during infection, including the synthesis of lipopolysaccharides and degradative enzymes, phytotoxins, antioxidants against ROS reactions, the secretion of effectors through the T3SS or manipulating hormone synthesis (Abramovitch *et al.*, 2006; Macho, 2016; Pfeilmeier *et al.*, 2016a; Albus *et al.*, 2001).

In addition to the knowledge acquired in recent years concerning how plant pathogenic bacteria evade plant immunity, there is still a gap in the current understanding of how these evasion mechanisms are regulated and the hierarchical and temporal control of these mechanisms (Pel and Pieterse, 2013; Le Fevre *et al.*, 2015; Pfeilmeier *et al.*, 2016a). Recent studies have demonstrated a role for the secondary messenger cyclic di-GMP (c-di-GMP) in the regulation of the elicitation of the plant defences (Freeman, 2008; Pfeilmeier *et al.*, 2016a; Pfeilmeier *et al.*, 2016b;). Secondary messengers function as signalling molecules and play important roles in the control of bacterial virulence (Fig. 8.2) (Iriti and Faoro, 2007; Liang, 2015; Pfeilmeier *et al.*, 2016a). In the next sections, we will discuss the role of the secondary messenger c-di-GMP in the virulence of plant pathogenic bacteria with a special emphasis on the impact of c-di-GMP on the bacterial evasion of the plant immunity. We will also discuss the relevance of c-di-GMP in the integration of environmental cues into regulatory networks governing numerous behaviours, such as motility, biofilm formation, degradative enzyme secretion and T3SS expression, that ultimately help to overcome plant immunity.

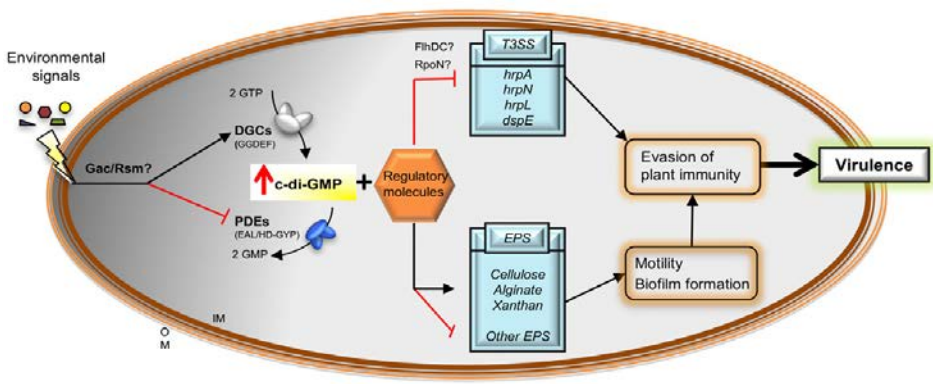

Figure 8.2 Regulation cascade of virulence factors by the secondary messenger cyclic di-GMP. Diguanylate cyclases (DGC) and phosphodiesterases (PDE) respond to different environmental signals through global regulatory two-component signal transduction systems, balancing intracellular levels of c-di-GMP. Specific regulatory molecules (proteins or RNAs riboswitches) bind, interact or sense c-di-GMP levels affecting the regulatory output (gene expression or enzymatic activity) of direct targets as, for example, the T3SS and exopolysaccharides, resulting in changes in the bacteria lifestyle and virulence.

Cyclic di-GMP: a relevant secondary messenger in pathogen–host interactions

C-di-GMP is a small diffusible secondary messenger discovered in 1987 as an allosteric activator of cellulose synthase in *Gluconacetobacter xylinum* (Ross *et al.*, 1987; Spoel and Dong, 2012). The history of c-di-GMP discovery has been well documented (Ross *et al.*, 1991; Jones and Dangl, 2006; Römling *et al.*, 2013). In the early 1980s, Benziman's group disclosed that cellulose synthase presented low activity without the secondary messenger (Aloni *et al.*, 1982). Further independent studies identified the cellulose synthase cofactor as bis (3′→5′) cyclic dimeric guanylic acid (c-di-GMP) (Ross *et al.*, 1987; Amikam and Benziman, 1989; Newman *et al.*, 2013). Several genetic studies identified, sequenced and characterized the genes encoding the enzymes responsible for c-di-GMP synthesis and degradation (Tal *et al.*, 1998; Hayashi *et al.*, 2001; Ausmees *et al.*, 2001; Simm *et al.*, 2004; Römling *et al.*, 2005; Tischler and Camilli, 2005). Specifically, two enzymes, the diguanylate cyclases (DGCs) and the phosphodiesterases (PDEs), which possess opposite activities, control c-di-GMP metabolism: (i) DGCs have a GGDEF domain and are responsible for the synthesis of c-di-GMP from two guanosine triphosphate molecules (2 GTP) and (ii) PDEs have an EAL or HD-GYP domain and generate two molecules of guanosine monophosphate (2 GMP) or a single phosphoguanylyl (3′–5′) guanosine molecule (pGpG) (Ross *et al.*, 1987; Ryan *et al.*, 2006; Sondermann *et al.*, 2012). The combined activity of these enzymes is responsible for the intracellular homeostasis of this secondary messenger, and numerous studies have inquired into the domains, function, activity and biochemistry of these two proteins (Fig. 8.2) (Chan *et al.*, 2004; Christen *et al.*, 2005; Malone *et al.*, 2007; Paul *et al.*, 2007; Henry and Crosson, 2011; Römling *et al.*, 2013; Wassmann *et al.*, 2007).

The c-di-GMP-signalling network connects specific environmental conditions (nutrient availability, oxygen, blue light, antibiotics, bile salts, among others) to the specific adaptive responses of bacteria, including the modification of gene expression, metabolism and bacterial lifestyle switches. The broad number of environmental signals that bacterial could sense is reflected on the diversity of signal-input domains observed in DGCs and PDEs proteins (Galperin *et al.*, 2001; Nürnberger *et al.*, 2004; Jenal and Malone, 2006; Hengge, 2009). These domains could include those reacting to light, such as the Blrp1 from *Klebsiella pneumoniae*, or gases, such as DosC and DosP from *Escherichia coli* (Barends *et al.*, 2009; Tuckerman *et al.*, 2009). The regulation of DGC, PDE and multicomponent response regulators by different environmental signals is well established and has been extensively reviewed (Hengge *et al.*, 2009; Krasteva *et al.*, 2012; Sondermann *et al.*, 2012).

The downstream signal transduction reflects the existence of a large variety of cytoplasmic proteins also known as c-di-GMP receptors, which specifically bind to c-di-GMP and translate the intracellular changes into specific outputs (Fig. 8.2) (Ausubel, 2005; Hengge, 2009). Several types of c-di-GMP receptors have been discovered, and the molecular details of c-di-GMP activity are beginning to be clarified: the PilZ domain proteins, transcriptional factors, enzymatically inactive DGC/PDE proteins and RNA riboswitches, are activated or repressed upon interaction with this secondary messenger (Weinhouse *et al.*, 1997; Hopkins and Sriskandan, 2005; Christen *et al.*, 2007; Pratt *et al.*, 2007; Vincent T Lee *et al.*, 2007; Hickman and Harwood, 2008; Hengge, 2009; Newell *et al.*, 2011; Srivastava *et al.*, 2011; Martínez-Granero *et al.*, 2014). However, recent investigations have demonstrated that c-di-GMP is able to bind to a large number of proteins without predictable binding domains as the membrane-bound PgaCD EPS synthase, the flagellar/type III export-ATPases FliI and

HrcN, the glutamyl ligase RimK and the histidine phosphatase/kinase CckA (Steiner *et al.*, 2013; Trampari *et al.*, 2015; Dubey *et al.*, 2016; Little *et al.*, 2016). This great variability of c-di-GMP binding proteins suggests that bioinformatics predictions based on predictable domains will fail in the identification of numerous important targets, therefore making further biochemical analysis essential. Given this vast diversity of effectors, regulation by the secondary messenger could occur at different levels (Sonderman *et al.*, 2012). The expression of numerous genes is controlled by transcriptional factors that will sense and bind to c-di-GMP as FleQ, which controls expression of flagella and adhesins in *Pseudomonas* sp. and Clp, which controls bacteria attachment in *X. campestris* (Hickman *et al.*, 2008; Tao *et al.*, 2010; Martínez-Gil *et al.*, 2014). Other receptors function at a post-translational level, involving protein–protein interactions, as YcgR from *E. coli* which has been demonstrated to interact and regulate the flagella motor (Boehm *et al.*, 2010).

The availability of the whole genome sequences of human-, animal- and plant- pathogenic bacteria has revealed the presence of numerous GGDEF, EAL and HD-GYP domain-containing proteins involved in c-di-GMP turnover, reinforcing the ubiquity and important role of this messenger in bacterial adaptation and lifestyle (Table 8.1) (Ausubel, 2005; Kulasakara *et al.*, 2006; Ryan *et al.*, 2007; Tamayo *et al.*, 2007; Bobrov *et al.*, 2011; Willmann *et al.*, 2011; Matas *et al.*, 2012). This secondary messenger has been implicated in the regulation of numerous bacterial behaviours, including biofilm formation (synthesis of adhesins and exopolysaccharides), flagellum-mediated motility, and virulence (Table 8.2). Bacterial processes associated with virulence and modulated by c-di-GMP signalling are typically associated with motility, adherence, invasion, cytotoxicity, intracellular infection, virulence factor secretion and immune response stimulation (Fig. 8.2) (Tischler and Camilli, 2005; Kulasakara *et al.*, 2006; Dow *et al.*, 2006; Hwee Siang Lee *et al.*, 2010; Moscoso *et al.*, 2011; Huang *et al.*, 2013; Moscoso *et al.*, 2014; Trda *et al.*, 2015; Trampari *et al.*, 2015). In particular, the association between bacterial motility, virulence and c-di-GMP has been widely studied. Flagella motility plays an essential role in effective plant colonization, migration into the apoplast and the plant vascular system. However, given that bacterial flagellin contributes to the induction of the early plant immune response, plant-associated bacteria needs to balance flagellar motility and their evasion of plant immunity. In several Pseudomonas, it has been shown that elevated levels of c-di-GMP reduce flagellin levels, helping the bacteria to evade the FLS2-mediated immune response (Pfeilmeier *et al.*, 2016b). In *P. syringae* high levels of c-di-GMP decrease the expression of flagellin (Engl *et al.*, 2014). Interestingly, this evasion of the FLS2-mediated immunity does not entail increase virulence, indicating that c-di-GMP controls several virulence-related phenotypes and the importance of motility for bacterial virulence. Interestingly, a recent study in numerous *Pseudomonas* species showed evidences that c-di-GMP is able to bind to FliI, the flagellum export ATPase and to HrcN, the homologue of the T3SS, suggesting a direct allosteric regulation by c-di-GMP (Trampari *et al.*, 2015).

The first observations of the role for c-di-GMP in bacterial virulence were reported in the animal pathogenic bacterium *Vibrio cholerae*, where low intracellular levels of c-di-GMP activated cholera toxin, and high levels of the secondary messenger attenuated virulence in an infant mouse model (Tischler and Camilli, 2005). Since these pioneering observations, numerous studies using genetic screens, overexpression approaches, or transcriptional profiling have implicated c-di-GMP levels in virulence regulation in a wide number of human pathogens, such as *Pseudomonas aeruginosa*, *Legionella pneumophila*, *Burkholderia*

Table 8.1 Enzymes involved in cyclic di-GMP metabolism (containing GGDEF and EAL/HD-GYP domains) in representative plant pathogenic bacteria

Organism	Disease	Genome size (bp)	Total proteins	C-di-GMP metabolism proteins	Enzymes described (references)	GenBank reference
Erwinia amylovora ATCC49946	Fire blight in fruit trees	3,795,810	3315	13 (Edmunds *et al*., 2013)	EdcA, EdcC and EdcE (Edmunds *et al*. 2013)	FN666575
Xylella fastidiosa 9a5c	Pierce's disease in grapevines	2,555,410	2141	5 (de Souza *et al.,* 2013)	CgsA, PD1671, EaL (Chatterjee *et al*., 2010; Cursino *et al*., 2015; de Souza *et al*., 2013)	AE003849
Dickeya dadantii Ech586	Soft rot disease in crops	4,870,170	4105	22 (He and Chou, 2015)	EcpB, EcpC and CsrD (Yi *et al*., 2010; Wu *et al*., 2014; Yuan *et al*., 2015)	CP002038.1
Ralstonia solanacearum GMI1000	Wilt disease in crops	5,567,800	4479	37 (He and Chou, 2015)	–	AL646052
Xanthomonas campestris pv. campestris ATCC33913	Black rot in crucifers	5,025,750	409	44 (Agrios, 2005)	RpfG (Ryan *et al*., 2006; Ryan *et al*., 2007)	AE008922
Xanthomonas oryzae pv. oryzae PXO99A	Leaf blight in rice	479,432	3736	30 (Agrios, 2005)	DgcA, GdpX1 (Su *et al.*, 2016; Yang *et al.*, 2016)	CP000967
Pseudomonas syringae pv. tomato DC3000	Bacterial speck disease	6,150,270	5255	56 (He and Chou, 2015)	BifA, Chp8 (Aragón *et al.*, 2015a; Engl *et al*., 2014)	AE016853
Pseudomonas savastanoi pv. savastanoi NCPPB3335	Olive knot disease	6,052,810	5098	34 (Rodriguez-Palenzuela *et al*., 2010)	PleD, BifA, DgcP (Pérez-Mendoza *et al*., 2014; Aragón *et al*., 2015a, 2015b	CM001834.1

Table 8.2 C-di-GMP specific sensors and effectors in representative plant pathogenic bacteria

Organism	Host	Sensor/effector	Motif/domain	Phenotypic output	Regulation level	Reference
Dickeya dadantii	*Solanaceae*	YcgR	PilZ	Regulation of T3SS (*hrpA*)	Transcriptional	Yuan *et al*., 2015
		EcpC and EGcpB	PDE	Positive regulation of T3SS (*hrpA*, *hrpN* and *dspE*)	Transcriptional	Yi *et al*., 2010
Pseudomonas syringae pv. tomato	Tomato plants	Chp8	DGC	Decreases flagellin and induces EPS	Unknown	Engl *et al*., 2014
		PleD	DGC	Negative regulation of *hrpL* and *hrpA*	Transcriptional	Pérez-Mendoza *et al*., 2014
Erwinia amylovora	Fruit trees	EdcC and EdcE	DGC	Induces amylovoran and biofilm formation; negative regulation of flagellar motility	Unknown	Edmunds *et al*., 2013
				Negative regulation of *hrpA*	Transcriptional	Edmunds *et al*., 2013
Pseudomonas savastanoi pv. savastanoi	Olive plants	DgcP	DGC	Negative regulation of motility and positive regulation of biofilm formation	Unknown	Aragón *et al*., 2015b
		BifA	PDE	Positive regulation of swimming motility; negative regulation of biofilm formation	Unknown	Aragón *et al*., 2015a
Xanthomonas oryzae	Rice	GdpX1	DGC	Negative regulation of T3SS, EPS and motility	Transcriptional	Yang *et al*., 2016
		DgcA	DGC	Negative regulation of EPS and motility; positive regulation of biofilm formation	Unknown	Su *et al.*, 2016
Xanthomonas campestris	Crucifers	RsmA	Transcriptional regulator	Negative regulation of biofilm formation	Post transcriptional	Lu *et al*., 2012
		YaqJ	C-di-GMP binding protein	Biofilm formation and virulence	Transcriptional/ protein–protein interaction	An *et al.*, 2014
Xylella fastidiosa	Fruit trees	CsgA	DGC	Negative impact in EPS and biofilm formation	Unknown	Chatterjee *et al*., 2010

DGC, diguanylate cyclase; EPS, exopolysaccharides; PDE, phosphodiesterase; T3SS, type III secretion system.

pseudomallei, *Brucella melitensis* and pathogenic *E. coli* (Tamayo *et al.*, 2007; Hwee Siang Lee *et al.*, 2010; Dodds and Rathjen, 2010; Levi *et al.*, 2011; Römling *et al.*, 2013; Peterson and Artis, 2014; Ishikawa *et al.*, 2014).

The role of c-di-GMP signalling in pathogenicity and virulence of plant pathogenic bacteria has been poorly treated compared with animal pathogens. Recent investigations of the plant pathogenic bacteria *X. campestris* pv. *campestris*, *P. syringae* pv. *tomato* and *P. savastanoi* pv. *savastanoi* revealed that DGCs and PDEs proteins are linked to a wide range of virulence phenotypes. The deletion of PDEs is frequently associated with a decrease in virulence, whereas the deletion of DGCs is associated with an increase in virulence (Ryan *et al.*, 2007; Yi *et al.*, 2010; Chatterjee *et al.*, 2010; Pérez-Mendoza *et al.*, 2011; Pel and Pieterse, 2013; Edmunds *et al.*, 2013; Aragón *et al.*, 2015a,b; Chin *et al.*, 2010). Nevertheless, the relationship between c-di-GMP sensors and effectors and bacterial virulence is more complex than previously anticipated, and several authors have reported a reduction in the virulence of various pathogenic bacteria as a consequence of the overexpression of some DGCs or the loss of function of either PDEs or DGCs. Currently, there is abundant evidence supporting an important role for c-di-GMP in bacterial virulence through effects on diverse bacterial regulation pathways in a complex interaction between DGC, PDE and the regulatory targets of c-di-GMP.

Cyclic di-GMP controls the expression of the type III secretion system

Consistent with many Gram-negative animal pathogens, the virulence of numerous phytopathogens, such as *P. syringae*, *X. campestris*, *Erwinia* species, *P. savastanoi*, *Ralstonia solanacearum* and *Pantoea stewartii*, relies on the assembly of the T3SS, a needle-like appendage that delivers a variety of virulence factors, called effectors, into the host cells (Fig. 8.1) (Tang *et al.*, 2006; Perez-Martinez *et al.*, 2010; Wenting Li *et al.*, 2014; Matas *et al.*, 2014; Wu *et al.*, 2015; Castañeda-Ojeda *et al.*, 2016; Popov *et al.*, 2016). Genes encoding the T3SS have been identified in *hrp* (hypersensitive response and pathogenicity)/*hrc* (hypersensitive response and conserved) gene clusters, which are essential for the translocation of effectors and the development of symptoms and diseases in host plants or the induction of the HR in non-hosts (Arnold *et al.*, 2003). The majority of T3SS effectors suppress plant immunity, particularly the signalling pathways triggered by flagellin and the elongation factor EF-Tu (Block and Alfano, 2011; Guttman *et al.*, 2014; Sun *et al.*, 2015). For more information on the functionality of the T3SS and its effectors in the modulation of plants, animals and human immunity readers are encouraged to consult these other reviews (McKinney, 2004; Alfano and Collmer, 2004; Tang *et al.*, 2006; Win *et al.*, 2013; Galán *et al.*, 2014; Pearson *et al.*, 2015). In addition, recent studies have demonstrated that T3SS-related effectors might play additional roles in the direct suppression of the plant immune system, such as contributing to bacterial survival, replication and dissemination by the modulation of plant hormone signalling, or metabolism and organelle function, all of which ultimately manipulate the host response (Guangyong Li *et al.*, 2014; Kang *et al.*, 2014; Kazan and Lyons, 2014; Macho, 2016). In addition to the *hrp/hrc* and T3SS effector genes, bacteria co-express a variety of other virulence-related genes that help bacteria to survive under the conditions encountered within the host (Yang *et al.*, 2004; Lam *et al.*, 2014). The expression of these genes is tightly regulated by a variety of environmental conditions (temperature,

medium composition, pH), host factors, surface sensing and plant-specific signals (Rahme *et al.*, 1992). In the phytopathogen *R. solanacearum* the expression of the T3SS is induced upon contact with host cells, similarly to the animal pathogen *Yersinia enterocolitica*. However, and differently from animal pathogens, this induction of the T3SS is not dependent on the T3SS pilus (Stainier *et al.*, 1997; Aldon *et al.*, 2000).

The effect of the secondary messenger c-di-GMP on T3SS expression was initially described in the human pathogen *P. aeruginosa* (Kulasakara *et al.*, 2006). Other studies in *P. aeruginosa* have demonstrated that c-di-GMP signalling, particularly the DGC SadC, coordinates the switch between the T3SS and T6SS via the RetS/GacS/LadS pathway (Moscoso *et al.*, 2011; Moscoso *et al.*, 2014). The mechanism of this regulation in *P. aeruginosa* is still under intense scrutiny, and novel results have demonstrated that c-di-GMP represses T3SS expression through intracellular levels of cAMP and the activity of Orn, a 3′ to 5′ exonuclease required for the full activation of the T3SS (Almblad *et al.*, 2015; Chen *et al.*, 2016). Further studies have revealed similar results in the human pathogens *V. cholerae* and *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) (Ahmad *et al.*, 2011; Roelofs *et al.*, 2015). In *S.* Typhimurium, high levels of c-di-GMP abolished the pro-inflammatory immune response and reduced the invasion phenotype in epithelial cells by the repression of the T3SS effector SipA (Lamprokostopoulou *et al.*, 2010; Ahmad *et al.*, 2011). Yi and colleagues (2010) demonstrated the role of c-di-GMP in T3SS expression in *Dickeya dadantii*, a plant phytopathogen responsible for the necrosis, blight and 'soft rot' disease in potato, celery, bulbs of many other vegetables and a variety of ornamental crops. Two PDE proteins, EGcpB and EcpC, from the 18 GGDEF/EAL domain proteins present in the genome, positively impact T3SS expression via the sigma factor HrpL, affecting *D. dadantii* virulence (Yi *et al.*, 2010). Particularly, c-di-GMP levels affect the transcription of the *hrpA*, *hrpN*, *dspE* and *hrpL* genes, which encode the major structural component of the T3SS pilus, a hairpin, an effector and an alternative sigma factor, respectively. Additional studies have demonstrated that $YcgR_{3937}$, a PilZ-domain protein from *D. dadantii* is required for T3SS regulation by EGcpB both *in vivo* and *in vitro* (Yuan *et al.*, 2015). Specifically, $YcgR_{3937}$ was demonstrated to bind c-di-GMP and impair *hrpA* expression. Simultaneously, *hrpL* expression is controlled by the flagella master regulator FlhDC, upon the induction of EcpC via the alternative sigma factor RpoN. Interestingly, EGcpB and EcpC contribute differently to T3SS expression, suggesting that each protein might play specific roles under certain environmental conditions (Yi *et al.*, 2010; Yuan *et al.*, 2015). The intracellular c-di-GMP effector YcgR is conserved in *Enterobacteriaceae*, although in *E. coli* and *S.* Typhimurium, this effector protein has been exclusively associated with bacterial motility (Ryjenkov *et al.*, 2006; Zorraquino *et al.*, 2013). YhjH, the EcpC homologue in *E. coli*, is activated through FliA differently from *D. dadantii* (Pesavento *et al.*, 2008).

Similar to *D. dadantii*, high levels of c-di-GMP inhibit the expression of *hrpA* in *E. amylovora*, the causative agent of fire blight disease in plants from the *Rosaceae* family. However, the global effect of the secondary messenger in *E. amylovora* virulence is complex and varies depending on the infection stage as follows: at early stages, low levels of c-di-GMP induce the expression of T3SS genes, which are required for initial host colonization; however, the subsequent bacterial establishment in the host vascular tissues requires high levels of c-di-GMP that increase the production of exopolysaccharide and the synthesis of degradative enzymes (Edmunds *et al.*, 2013; Piqué *et al.*, 2015).

The bacterial plant pathogen *P. syringae*, responsible for major disease outbreaks in fruit trees, rice, tomatoes, corn, cucumber and beans, also uses a T3SS to deliver virulence factors and promote colonization and infection (Anderson *et al.*, 2014). In *P. syringae* pv. tomato and pv. phaseolicola, the overexpression of the DGC PleD leads to a 2-fold decrease in the expression of *hrpL* and *hrpA* genes, encoding the alternative sigma factor and protein subunits of the T3SS, respectively. However, this reduction in T3SS expression apparently does not affect bacterial virulence *in vivo* (Pérez-Mendoza *et al.*, 2014). On the contrary, the overexpression of PleD in *P. savastanoi* does not inhibit *hrpL* and *hrpA* expression, although it clearly affects the symptomatology of the disease (Pérez-Mendoza *et al.*, 2014). The DGC DgcP, a conserved protein in Pseudomonads, has been associated with an increase in the intracellular levels of c-di-GMP and the reduction of the virulence of *P. aeruginosa* PAK and *P. savastanoi* pv. savastanoi NCPPB3335 in an acute lung injury model and 1-year-old olive plants, respectively (Aragón *et al.*, 2015b). Moreover, the overexpression of DgcP repressed the T3SS and induced the T6SS in *P. aeruginosa*, consistent with the previous observations of Moscoso and colleagues (2011). Recent findings in *X. oryzae* demonstrated the negative impact of the DGC GdpX1 in *hrpG*, *hrpX* and *hpa1* mRNA levels and other virulence factors, such as exopolysaccharide production and motility (Yang *et al.*, 2016).

These apparently contradictory findings suggest that a highly complex regulation pathway is mediated by the secondary messenger c-di-GMP and specific modulatory enzymes, which introduce diversification in the final regulation of T3SS expression in related and non-related bacteria.

Cyclic di-GMP coordinates exopolysaccharide production

Plant-associated bacteria form surface communities known as biofilms. Within a biofilm, bacterial cells are tightly associated via an extracellular matrix that provides protection against changing environmental conditions, such as desiccation and radiation (Flemming and Wingender, 2010). Any of the plants' surfaces, aerial tissues, vascular system and surrounding plant roots can potentially be covered with a biofilm of either epiphyte or endophyte bacteria, including pathogens or symbionts microbes (Ramey *et al.*, 2004; Danhorn and Fuqua, 2007; Castiblanco and Sundin, 2016). Indeed, the ability to form biofilms is a common strategy for the survival or transmission of pathogens (Koczan *et al.*, 2009; Chatterjee *et al.*, 2010), particularly in the xylem-dwelling pathogens *Erwinia amylovora*, *R. solanacearum*, *X. campestris* and *X. fastidiosa* (Fig. 8.1) (Koczan *et al.*, 2009; Chatterjee *et al.*, 2010; Castiblanco and Sundin, 2016).

Surface proteins, surface polysaccharides (SPSS), including exopolysaccharides (EPSs) and lipopolysaccharides (LPSs), and extracellular DNA are major structural components that constitute the extracellular matrix of bacterial biofilms (Flemming and Wingender, 2010). Interestingly, SPSS, EPSs and LPSs are among the major virulence factors in bacterial pathogens that help bacterial cells evade plant immunity (D'Haeze and Holsters, 2004). Plant pathogens produce a large number of different polysaccharides, as alginate, cellulose, levan, amylovoran, succinoglycan and xanthan (Sunish Kumar and Sakthivel, 2001; Kumar *et al.*, 2003; D'Haeze *et al.*, 2004; Laue, 2006; Koczan *et al.*, 2009; Vrancken *et al.*, 2013; Pérez-Mendoza *et al.*, 2014; Aragón *et al.*, 2015a; Castiblanco and Sundin, 2016; Ordax *et al.*, 2010). Reflecting their highly hydrated and anionic characteristics, EPSs contribute to bacterial fitness and interactions with plants in a multifaceted manner, involving resistance

to stress tolerance, colonization, growth, and survival in plants and an increase in bacterial pathogenicity and virulence (Fig 8.1) (Denny, 1995; D'Haeze *et al.*, 2004; Ordax *et al.*, 2010). Moreover, recent findings have suggested that SPSS could contribute to the differential host specificity of closely related plant pathogenic bacteria (Kawaharada *et al.*, 2015). During infection, EPSs could help bacterial cells escape from plant defences or suppress basal plant responses (Pellock *et al.*, 2000; D'Haeze and Holsters, 2004; Büttner and Bonas, 2010; Aslam *et al.*, 2008). As previously described, plant responses involve the massive production of ROS, antibiotics and antibacterial cationic peptides, thus it has been proposed that both SPSS and EPSs might protect bacterial cells from the plant immunity in the following three different ways: (i) sequestering antibiotics and antimicrobial compounds in different compartments; (ii) forming electrostatic barriers to repel cationic antimicrobial compounds; and (iii) acting as a physical diffusion barrier against ROS (D'Haeze *et al.*, 2004).

Studies with *X. campestris* have demonstrated that xanthan and cyclic glucan EPSs suppress callose deposition in tobacco and *A. thaliana* plants, thereby increasing the susceptibility of plants and the progression of the diseases (Yun *et al.*, 2006; Rigano *et al.*, 2007). *Xanthomonas* polysaccharides also chelate calcium ions present in plant apoplasts, which is essential for the activation of the PTI response (Aslam *et al.*, 2008). The vascular pathogen *E. amylovora* produces two types of EPSs, levan and amylovoran, which are both involved in biofilm formation, virulence and pathogenicity (Koczan *et al.*, 2009). Both molecules also mediate survival under certain environmental conditions and likely contribute resistance to anti-microbial compounds produced from plants during infection (Ordax *et al.*, 2010). Interestingly, recent findings revealed that amylovoran production and T3SS expression are negatively regulated by two-component signal transduction systems EnvZ/OmpR and GrrS/GrrA (Wenting Li *et al.*, 2014). Interestingly, some EPSs induce the host immune system. Two examples of this phenomena are as follows: (i) the EPS I from the plant pathogen *R. solanacearum*, which specifically induces the defence response in wilt-resistant tomato plants; and (ii) the EPS from *X. campestris* pv. *vesicatoria*, which induces the production of the broad-spectrum antimicrobial molecule phytoalexin (Romeiro and Kimura, 1997; D'Haeze *et al.*, 2004; Milling *et al.*, 2011).

Phytopathogenic bacteria have evolved sophisticated regulatory systems to adjust the synthesis of EPSs to extracellular conditions, such as nutrient availability and the presence of plant-derived molecules (Vojnov *et al.*, 2001; He *et al.*, 2009). Two-component signal transduction systems, stress response mechanisms, sigma factors, and post-transcriptional regulators constitute the regulatory network of polysaccharide synthesis during plant infection (He and Zhang, 2008; Büttner and Bonas, 2010). In many pathogenic or commensal bacterial species, c-di-GMP levels control EPS production in different ways (Steiner *et al.*, 2013; Edmunds *et al.*, 2013; Cursino *et al.*, 2015; Pérez-Mendoza *et al.*, 2015; Aragón *et al.*, 2015b; Köseoğlu *et al.*, 2015; Pérez-Mendoza and Sanjuán, 2016; Yang *et al.*, 2016). Recent studies on bacterial plant pathogens from the *P. syringae* complex have implicated c-di-GMP signalling in the virulence of these phytopathogens (Pérez-Mendoza *et al.*, 2014; Aragón *et al.*, 2015a; Aragón *et al.*, 2015b; Pfeilmeier *et al.*, 2016a). Furthermore, the active DGC DgcP and the PDE BifA are highly conserved among bacteria from the genus *Pseudomonas*, including the opportunistic pathogen *P. aeruginosa*. Particularly, the overexpression of DgcP positively impacts EPS production, although *dgcP* mutants of either *P. savastanoi* and *P. aeruginosa*, are arrested in virulence (Aragón *et al.*, 2015b). The overexpression of PleD, a DGC

enzyme, from *Caulobacter crescentus*, did not influence the virulence of *P. syringae* pv. tomato or *P. syringae* pv. phaseolicola; however, this enzyme increases the knot size and reduces the plant necrosis induced by *P. savastanoi* pv. savastanoi (Pérez-Mendoza *et al.*, 2014). The DGC Chp8 from *Pseudomonas syringae* pv. tomato DC3000 is responsible for the increase in c-di-GMP levels during early stages of infection, which decreases flagellin synthesis and increases EPS production, cooperatively contributing to effective bacterial evasion from the plant immunity (Engl *et al.*, 2014). Interestingly, Engl and collaborators (2014) showed that Chp8 is encoded within the *hrp* cluster regulon, although its expression is independent of HrpL. This study have also shown that phloretin, a plant flavonoid produced upon bacterial infection, induces *chp8* expression with the consequent increase in c-di-GMP levels and the production of the EPS (Engl *et al.*, 2014).

Although it is commonly assumed that the increase in the secondary messenger c-di-GMP induces EPS synthesis, biofilm formation and bacterial cell autoaggregation, the connection between these processes is complex and depends on the bacterial species and its adaptation to host signals and the environment. In *E. amylovora* pathogenesis, biofilm formation and the amylovoran EPS are essential for vascular tissue colonization and virulence. The synthesis of this EPS is regulated by two DGCs: EdcC and EdcE (Ayers *et al.*, 1979; Edmunds *et al.*, 2013). The double mutant *edcCE* showed low levels of c-di-GMP and was impaired in biofilm formation and amylovoran production, but surprisingly, showed increased virulence, most likely affecting the expression of additional virulence factors as discussed above (Edmunds *et al.*, 2013). This outcome could reflect the fact that *E. amylovora* pathogenesis is complex and still poorly understood. However, this finding also indicates that different virulence factors might be essential for early stages of infection, while other factors might be required only during subsequent periods. In *X. oryzae* pv. *oryzae*, the DGC DgcA negatively affects EPS production, motility, autoaggregation and virulence against two different rice cultivars, although positively contributing to biofilm formation (Su *et al.*, 2016). Nevertheless, high levels of c-di-GMP in *X. fastidiosa* repress both EPS synthesis and biofilm formation (Chatterjee *et al.*, 2010; de Souza *et al.*, 2013). Chatterjee and colleagues (2010) demonstrated that the DGC CsgA negatively impacts EPS production and biofilm formation and promotes bacterial migration through the vascular system of the plant, thereby causing disease.

Currently, c-di-GMP has been implicated in the regulation of more than ten different EPSs from plant and animal pathogens at different levels (Pérez-Mendoza and Sanjuán, 2016). Studies have highlighted that bacterial EPSs have diverse functions in bacterial cells during interactions with the host and are key elements in the cross talk between bacteria and plants or animal hosts, which determines the outcome of a given interaction.

Conclusions and future perspectives

Approximately 100 bacterial species cause plant diseases and a variety of symptoms from leaf spots and blights to soft rots, wilts, knots and cankers (Agrios, 2005). The diversity of the symptomatology relies on the arsenal of different virulence mechanisms and regulatory pathways engaged in pathogenesis. In recent years, the secondary messenger c-di-GMP has emerged as a universal key molecule in the control and regulation of virulence in plant pathogenic bacteria. However, the multiplicity of single species of DGCs, PDEs enzymes and c-di-GMP-binding effector proteins has cluttered the comprehension of c-di-GMP

signalling. The multiple divergences observed in different bacterial species regarding c-di-GMP regulation could be explained by the research approach based on the overexpression or mutation of specific DGCs or PDEs and further evaluation of its functionality on global virulence, instead of particular virulence-related phenotypes in a temporal frame of reference.

To acknowledge the importance of c-di-GMP in the virulence of plant pathogenic bacteria, future investigations should commit to determining the specific molecular and physiological functions of all of the enzymes involved in c-di-GMP metabolism in a temporal-spatial context within the infection process. The following are some relevant questions that should be addressed to fully comprehend the intricate pathway of c-di-GMP signalling: (i) what is the nature of the signals responsible for the activation or repression of DGCs and PDEs within the infection process?; (ii) what is the specific function of these enzymes in the cell within a time and space during different infection stages; or (iii) how is the cross-talk between the diverse enzymes and the enzymatic network interconnected with other global regulatory networks operating in bacteria? The understanding of this close molecular relationship between bacterial pathogens and their hosts could contribute to the development of more pathogen resilient crops and improve strategies for crop development.

Conflicts of interest disclosure

The authors declare no competing financial interests.

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