Appl Microbiol Biotechnol (2010) 87:467–477
DOI 10.1007/s00253-010-2631-2

MINI-REVIEW

Bacteria causing important diseases of citrus utilise distinct modes of pathogenesis to attack a common host

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Received: 21 February 2010 / Revised: 14 April 2010 / Accepted: 15 April 2010 / Published online: 7 May 2010

Abstract In this review, we summarise the current knowledge on three pathogens that exhibit distinct tissue specificity and modes of pathogenesis in citrus plants. *Xanthomonas axonopodis* pv. *citri* causes canker disease and invades the host leaf mesophyll tissue through natural openings and can also survive as an epiphyte. *Xylella fastidiosa* and *Candidatus* Liberibacter are vectored by insects and proliferate in the vascular system of the host, either in the phloem (*Candidatus* Liberibacter) or xylem (*X. fastidiosa*) causing variegated chlorosis and huanglongbing diseases, respectively. *Candidatus* Liberibacter can be found within host cells and is thus unique as an intracellular phytopathogenic bacterium. Genome sequence comparisons have identified groups of species-specific genes that may be associated with the particular lifestyle, mode of transmission or symptoms produced by each phytopathogen. In addition, components that are conserved amongst bacteria may have diverse regulatory actions underpinning the different bacterial lifestyles; one example is the divergent role of the Rpf/DSF cell–cell signalling system in *X. citri* and *X. fastidiosa.* Biofilm plays a key role in epiphytic fitness and canker development in *X. citri* and in the symptoms produced by *X. fastidiosa*. Bacterial aggregation may be associated with vascular occlusion of the xylem vessels and symptomatology of variegated chlorosis.

Keyword Citrus

Introduction

Xanthomonas axonopodis pv. *citri*, *Xylella fastidiosa* and *Candidatus* Liberibacter *asiaticus* are bacteria that infect citrus plants, causing citrus canker disease (CCD), citrus variegated chlorosis (CVC) and huanglongbing (HLB),

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respectively. These diseases greatly reduce production, eventually causing death of the infected trees, with substantial economic impact. HLB is the most destructive disease of citrus worldwide (Bove 2006; Stokstad 2006; Callaway 2008) and because of the major outbreaks of the HLB that started in Brazil (in 2004) and USA (in 2005), these countries have already removed tens of millions of trees, with an impact of billions of dollars since its occurrence alone. *X. citri* and *X. fastidiosa* are related organisms that belong to the γ subdivision of the Proteobacteria in the family of Xanthomonadaceae. *Ca.* Liberibacter *asiaticus* in contrast belongs to the order *Rhizobiales* in the α-subdivision of the Proteobacteria (Doddapaneni et al. 2008) and is closely related to members of the *Rhizobiaceae* family (Duan et al. 2009).

Although these bacteria attack a common host, they adopt different strategies of pathogenesis and dissemination, show different tissue specificity and cause different symptoms. *X. citri* invades the host leaf mesophyll tissue through natural openings such as stomata and through lesions and subsequently proliferates in the intercellular spaces. The bacterium can also survive and multiply outside the host as an epiphyte. On the other hand, *X. fastidiosa* and *Candidatus* Liberibacter are vectored by insects and proliferate in the vascular system of the host, where they are limited to either the phloem (*Candidatus* Liberibacter) or xylem (*X. fastidiosa*). *Candidatus* Liberibacter is unique amongst plant pathogenic bacteria in that it occurs within plant cells.

There is a great deal of interest in understanding the molecular mechanisms of pathogenesis of these three major pathogens of citrus plants as a route to identification of new strategies for disease management. The economical and social impact of these diseases to the citrus industry encouraged the sequencing of the complete genome of all three pathogens (Simpson et al. 2000; da Silva et al. 2002; Duan et al. 2009). Indeed, *X. fastidiosa* was the first plant pathogenic bacterium to be sequenced (Simpson et al. 2000). The determination of the genome sequence of *Ca.* Liberibacter *asiaticus* presented a particular problem since conditions for the cultivation of this fastidious organism and the related *Candidatus* L. *americanus* and *Candidatus* L. *africanus* were only very recently reported (Sechler et al. 2009). The full genome sequence of *Ca.* L. *asiaticus* was determined using a metagenomic approach on DNA extracted from a single insect vector or from the phloem of an HLB-infected tree (Duan et al. 2009; Tyler et al. 2009).

Comparative genomic studies have identified a number of genes and operons that might be relevant to the adaptation to citrus, interactions with insects and to tissue specificity. In addition to these genomic studies, work to identify virulence factors in *X. citri* and *X. fastidiosa* has been informed by investigations on other xanthomonads such as the brassica pathogen *Xanthomonas campestris* pv. *campestris* and rice pathogen *Xanthomonas oryzae* pv. *oryzae.* In this review, we present an overview of the biology of each of the pathogens, the diseases they cause and current management practices, comparative genomics of the three citrus pathogens and discuss existing knowledge of virulence mechanisms and their regulation, with an emphasis on cell–cell signalling. The roles of secretion systems, biofilm formation and attachment are also included.

General features of the bacterial diseases in citrus and economic impacts

Citrus canker

CCD is caused by five different groups of *X. citri* strains with variation in host range: three from Asia (A, A* and Aw) and two that form a phylogenetically distinct group originated in South America (B and C). The Asiatic (A) group (*X. citri* pv. *citri* A) has the widest host range and is widespread throughout the world. The B and C groups (*X. citri* pv. *aurantifolii* B and C) have only been found in South America and have a reduced host range compared with the A groups (Stall and Seymour 1983). New groups of *X. citri* pv. *citri* (Aw from Florida and A* from Southwest Asia) were found primarily on Mexican lime (*Citrus aurantifolia*) and are restricted in host range and pathogenicity (Verniere et al. 1998; Sun et al. 2004).

Canker symptoms on leaves and fruit are characterised by surface-penetrating necrotic lesions surrounded by oily, water-soaked margins and yellow chlorotic rings (Brunings and Gabriel 2003) (Fig. 1a). All cultivars of citrus are susceptible to canker and the bacteria entrance occurs mainly through stomata and wounds. Differences in leaf characteristics such as rapidity of development of the cuticle and the number and structure of stomata have been implicated in the differences between susceptible (grapefruit, Mexican lime and lemon) and moderately resistant (mandarins) plants. Infected fruits have decreased commercial qualities and are rejected by most important markets (Canteros 2004).

Two different lines of defence against CCD have been employed: spray application of antibiotics and copper-based products and eradication through removal of infected as well as the surrounding trees within an appropriate radius. The more to most effective way to eliminate the disease before it becomes endemic is through the removal of affected plants, a procedure that causes significant economical losses. In São Paulo State, the main citrus production area in Brazil, it is mandatory to eliminate all plants around the focus of infection in a

30-m radius if the contaminated plants are less than 0.5% of the planting block and all plants in the planted block if the index of contaminated plants is more than 0.5%. In the latter case, cultivation is then prohibited in the area for the next 3 years and there is no payment for lost production to the growers. In Florida, the eradication was considered infeasible due to the wide distribution caused by the hurricane season of 2004, then the programme was interrupted in 2006. CCD has a further great impact on the economy of the citrus industry in a specific country or region due to quarantine restrictions imposed by some importer countries (the so-called non-tariff trade barriers).

Citrus variegated chlorosis

X. fastidiosa is the causal agent of diseases of economically important crops such as grapevine, plum almond, peach, coffee and citrus (Purcell and Hopkins 1996; Lima et al. 1998). However, each disease is caused by a different strain of *X. fastidiosa*. The symptoms produced by *X. fastidiosa* in CVC disease are characterised by water stress produced by bacterial aggregates and production of extracellular polysaccharide (EPS) causing vascular occlusion in xylem vessels (de Souza et al. 2003). The most common symptoms are smaller and mottled fruits that are maturing together with reduced leaf size exhibiting a chlorosis similar to that seen in zinc deficiency (Fig. 1b). Young leaves do not show symptoms but as they mature, small light brown gummy lesions appear which in due time become dark brown or even necrotic. *X. fastidiosa* is transmitted amongst plants by leafhoppers (Hemiptera, Cicadellidae) or spittlebugs (Purcell and Hopkins 1996).

Huanglongbing

HLB is the disease of citrus formerly known as citrus greening. The name, which in Mandarin means the 'yellow shoot disease', refers to the presence of yellowish twigs in infected citrus plants (Fig. 1c). HLB is more destructive and devastating than previous citrus disease epidemics because of the lack of efficient control coupled with the insect-vectored mechanism of dissemination; great losses have been caused in citrus industries throughout the world (Hsiang 1956; Bové 2006). The disease was first detected in 1919 in China, subsequently reported in South Africa also in the early twentieth century and later spread to several countries in both continents (Zhao 1981; Bové 2006). HLB is characterised by the presence of yellow shoots, blotchy mottle on the leaves and fruits that are small and lop-sided, with inverted colouring. Three α-Proteobacteria species are associated with this citrus disease: *Ca.* Liberibacter *asiaticus* in Asia, *Ca.* L. *africanus* in Africa (Jagoueix et al. 1994) and *Ca.* L. *americanus* in Brazil, South America (Teixeira 2005). Although *Ca.* L. *asiaticus* does not always produce disease, it can invade the most rutaceous species and some solanaceous (Halbert and Manjunath 2004). Psyllid insects mediate transmission of the pathogen amongst plants. *Ca.* L. *asiaticus* and *Ca.* L. *americanus* are transmitted amongst citrus trees in Asia and America by *Diaphorina citri* whereas *Ca.* L. *africanus* is

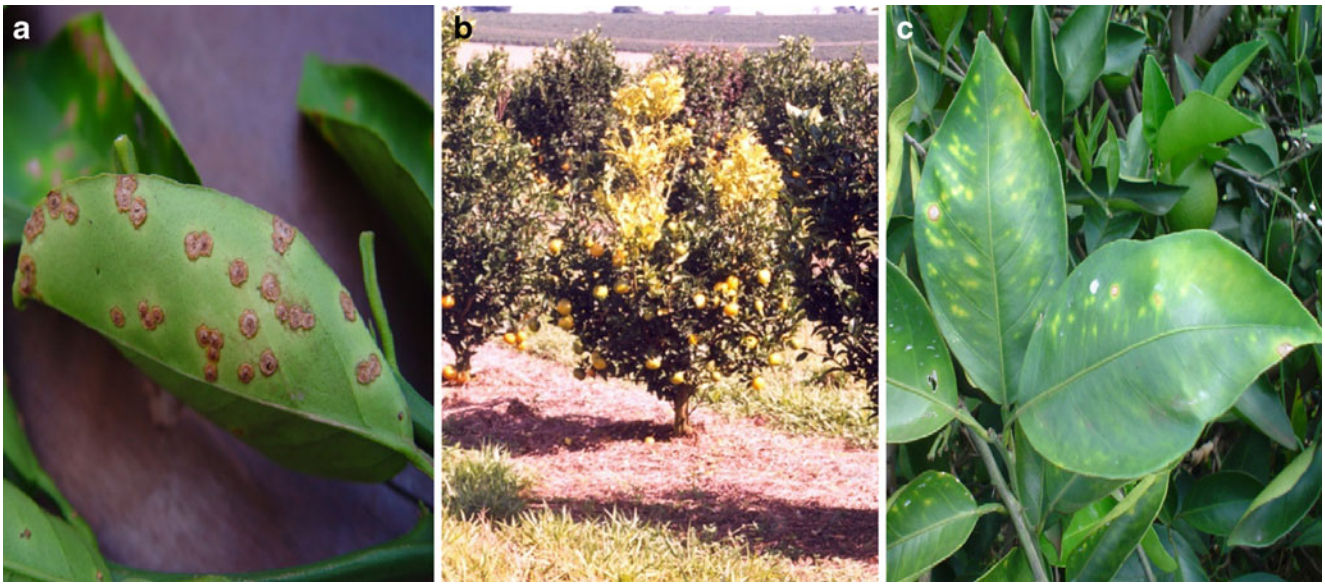

Fig. 1 Bacterial symptoms on citrus plants: lesions caused by citrus canker on citrus leaves (**a**), 'yellow shoot' symptoms of huanglongbing on citrus tree (**b**) and citrus variegated chlorosis (**c**)

transmitted in Africa by *Trioza erytreae* (McClean and Oberholzer 1965; Capoor et al. 1967; Bové 2006).

The unculturable characteristic of *Candidatus* Liberibacter has represented a major obstacle towards the advancement in the knowledge of HLB disease over the past century. In spite of this limitation, the entire genome sequence of '*Ca.* Liberibacter *asiaticus*' has been obtained by pyrosequencing technologies on DNA extracted from a single '*Ca.* L. *asiaticus*'-infected Asian citrus psyllid (*D. citri*) (Duan et al. 2009) and from phloem tissue of citrus trees infected with HLB (Tyler et al. 2009). This is the first genome sequence of an α-Proteobacterium that acts as both an intracellular plant pathogen and an insect symbiont. A very recent report describes the cultivation of *Candidatus* Liberibacter in vitro in a medium used previously for spiroplasmas or *X. fastidiosa* growth supplemented with citrus vein extract (Sechler et al. 2009). This represents an important first step towards functional genomic analysis of virulence in this pathogen.

Comparative genomic analysis

The general features of the genomes of the three major citrus bacterial pathogens are summarised in Table 1. The genome sequence of *X. citri* comprises 5,175,554 bp of chromosomal sequence and two plasmid sequences (64,920 and 33,699 bp) whereas the other two bacteria show much more reduced genome content. *X. fastidiosa*, which is related to *X. citri*, comprises 2,679,305 bp of chromosomal sequence and two plasmids (51,158 and 1,285 bp) whereas *Ca.* Liberibacter *asiaticus* has only one chromosome (1,227,204 bp in length) with no plasmids (Table 1). The reduced genome size of *X. fastidiosa* and *Ca.* Liberibacter *asiaticus* may reflect a lifestyle in which the bacteria are always intimately associated with either a plant or insect host. In contrast, the larger genome size of *X. citri* may reflect the added capability of this organism to grow outside the plant either as an epiphyte on plant leaf surfaces or in the soil in association with dead plant parts. It is important to note that *Ca.* Liberibacter *asiaticus* contains genetic features distinctive due to its obligate intracellular characteristic (Moran 2002), such as having a small genome size, a low GC content (36.5%) and a significant genome reduction compared to other members of the *Rhizobiaceae* family. The information revealed by this newly completed genome may make it possible to identify those conditions necessary for its growth in culture as well as aid in our understanding of how this pathogen becomes established in both its vector and plant hosts. This genome knowledge may also provide information relevant to two other genomes of HLB bacteria, *Ca.* Liberibacter *americanus* and *Ca.* Liberibacter *africanus*.

To compare the content of shared genes, a BLAST search amongst all predicted ORFs in the *X. citri*, *X. fastidiosa* and *Ca.* Liberibacter *asiaticus* genomes was performed. Reciprocal best matches were counted by a BLAST result with an expectation value *E* of <1e−10 each. A total of 96 protein clusters were conserved in all three genomes

Table 1 General genome features of the three major citrus bacterial pathogens *Xanthomonas citri* pv. *citri*, *Xylella fastidiosa* and '*Candidatus* Liberibacter *asiaticus*'

	X. citri	*X. fastidiosa*	*Ca.* Liberibacter *asiaticus*
Chromosome			
Length (bp)	5,175,554	2,679,305	1,227,204
G+C content (%)	64.7	52.7	36.5
Protein-coding genes	4,313	2,782	1,136
Hypothetical proteins	331	1,083	362
tRNA	54	49	44
Plasmids	2	2	0
Plasmid 1			
Length (bp)	64,920	51,158	–
G+C content (%)	61.4	49.6	–
Protein-coding genes	73	64	–
Hypothetical proteins	27	24	–
Plasmid 2			
Length (bp)	33,699	1,285	–
G+C content (%)	61.9	55.6	–
Protein-coding genes	42	2	–
Hypothetical proteins	14	1	–

(i.e. each cluster has at least one gene from each of the three species); the majority of these play a role in the housekeeping pathways of the cell. On the other hand, a total of 2,964 clusters of *X. citri*, 1,607 from *X. fastidiosa* and 654 from *Ca.* Liberibacter *asiaticus* were unique to those organisms (Fig. 2).

Genomic insights into virulence-related functions: secretion systems

Bacterial secretion systems are important for the interaction of pathogens with the host. They serve to deliver virulence determinants to the bacterial environment or within the host cell, can act in secretion of adhesins involved in attachment and biofilm formation and are involved in defence against antimicrobial agents of host origin.

Type I secretion systems are involved in the secretion of toxins such as haemolysins and in the import and efflux of a variety of compounds including drugs. All three pathogens appear to have such systems. Type II secretion systems (T2SS) of plant pathogenic bacteria secrete a number of possible virulence determinants into the bacterial environment, including enzymes with plant cell wall-degrading activity. *X. citri* has two different T2SS (encoded by the *xps* and *xcs* gene clusters), a large number of cell wall-degrading enzymes and sugar transporters, which may act in taking up the products of enzymatic degradation of plant polysaccharides. In contrast, *X. fastidiosa* has only one T2SS, (the Xps system) and *Ca.* Liberibacter *asiaticus* has no T2SS. The *X. fastidiosa* type II secretion system is probably involved in the export of cell wall degrading enzymes, including endoglucanase, polygalacturonase and perhaps several proteases. One function of these enzymes in *X. fastidiosa* pathogenesis is to degrade the pit membranes, allowing bacteria to move into previously uncolonised xylem vessels (Roper et al. 2007; Perez-Donoso et al. 2010). Other roles may be to mobilise cell walls for nutritional purposes or to overcome plant defences. Unlike *X. citri* and *X. fastidiosa*, *Ca.* L. *asiaticus* lacks genes for these extracellular degradative enzymes.

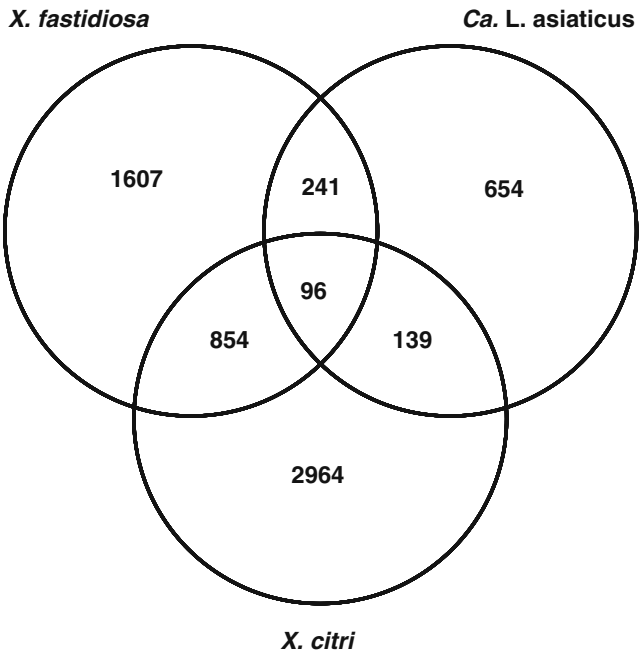

Fig. 2 Venn diagram representing the number of clusters of related protein families. Comparative analysis of the *X. citri* (*Xac*), *Xylella fastidiosa* (*Xf*) and *Ca.* Liberibacter *asiaticus* (*CLas*) genomes, performed by using protein clustering algorithms

Type III secretion systems (T3SS) are very conserved amongst plant and animal pathogenic bacteria and act to deliver effector proteins from the bacterial cytoplasm into the host cell. These effectors can act as virulence factors, interfering with a variety of host functions to promote disease, although in some plants, specific effectors may be recognised to trigger defence-related responses. As many effectors were originally described through this latter function, they were termed avirulence determinants. The T3SS machinery is present in *X. citri* but absent in both *X. fastidiosa* and *Ca.* Liberibacter *asiaticus*. Of the effector genes in *X. citri*, *pthA*, a member of *avrBS3*/*pthA* genes family, plays the most important role in pathogenicity and is present in all *Xanthomonas* that causes canker in citrus, often in multiple copies (Swarup et al. 1992; Cubero and Graham 2002). AvrBs3/PthA family proteins contain particular structural features with 15 to 30 repeats of 34 amino acids in their central portion, a leucine zipper-like motif, nuclear localisation signals and an acid transcriptional activation domain in the C terminus (Zhu et al. 1998; Lahaye and Bonas 2001). The exact number and arrangement of their repeat units differ and contribute to function and specificity during the elicitation of resistance and virulence on the respective host species. The repeat region mediates direct binding to DNA (Kay et al. 2007) and the binding specificity is based on a two-amino acid motif per repeat (Boch et al. 2009). This binding allows effectors of the AvrBs3 family to mimic plant transcriptional activators and manipulate the plant transcriptome (Kay and Bonas 2009).

Recent screening of a transposon-based mutant library in Rangpur lime (*Citrus limonia*) plantlets identified two genes associated with the T3SS, *hrpB4* and *hrpX* having a role in the production of canker symptoms (Laia et al. 2009). HrpB4 is not secreted and may form part of the secretion machinery, whereas HrpX is a regulator of the expression of genes in the *hrp* regulon, which encode structural components of the secretion apparatus as well as some of the secreted effectors (Laia et al. 2009).

The type IV secretion system (T4SS) is also involved in transporting macromolecules from the bacterial cytoplasm

into the host cell cytoplasm. The best-studied T4SS is the *vir*-encoded system of *Agrobacterium*, which is involved in the transfer of the T-DNA. *X. citri* and *X. fastidiosa* have gene clusters similar to the T4SS of *Bordetella pertussis* which is involved in secretion of pertussis toxin (Van Sluys et al. 2002). By analogy, the T4SS systems in the citrus pathogens may also play a role in virulence. *Ca.* L. *asiaticus* lacks both type III and type IV secretion systems, which is consistent with its intracellular nature.

X. fastidiosa and *X. citri* contain genes for surface-associated adhesins that appear to be type V secretion system autotransporters. These adhesins are very large proteins (over 3,000 amino acids) that are thought to have a role not only in adhesion to biotic surfaces but also in biofilm formation. In *X. citri*, the gene encoding the transporter protein XacFhaC is closely linked to a gene encoding a filamentous hemagglutinin-like protein named XacFhaB (Gottig et al. 2009). Mutational analyses indicated that XacFhaB is involved in virulence and influences leaf surface attachment and biofilm formation. *X. fastidiosa* has several large proteins with amino acid sequence that are similar to XacFhaB, although similarity is restricted to domains located at the N and C termini. No proteins with related domains are seen in *Ca.* L. *asiaticus*.

As outlined above, genome sequence comparisons can identify groups of species-specific genes that may be associated with the particular lifestyle, mode of transmission or symptoms produced by each phytopathogen. Such comparisons can guide functional genomic analyses and prioritise genes for study. However, it is evident that, in addition to differences in gene complement, diverse regulatory actions of components that are conserved between bacteria may underpin different bacterial lifestyles. One example is the divergent role of the Rpf/DSF cell–cell signalling system in *X. citri* and *X. fastidiosa*, which we discuss in the following sections.

Conserved cell–cell signalling systems have different regulatory actions in *X. citri* and *X. fastidiosa*

Bacterial quorum sensing regulates gene expression in a cell-density-dependent manner through a synthesis of a variety of small signalling molecules (Whitehead et al. 2001; Bassler 2002). In *X. citri* and *X. fastidiosa*, as in other bacteria, cell-to-cell signalling plays an important role in colonisation, pathogenesis and biofilm formation (Siciliano et al. 2006; Chatterjee et al. 2008b). Both bacteria have a cell-to-cell signalling system mediated by a diffusible signal molecule called DSF. Genes within the *rpf* cluster (for regulation of pathogenicity factors) encode the components of the DSF signalling system (Barber et al. 1997; Newman et al. 2004; Chatterjee et al. 2008b). RpfF is responsible for the synthesis of DSF whereas RpfC and RpfG comprise a two-component system implicated in DSF perception and signal transduction (Tang et al. 1991; Dow and Daniels 1994; Barber et al. 1997; Slater et al. 2000; Chatterjee et al. 2008b). RpfC is a complex sensor kinase with an N-terminal membrane-associated input domain, histidine kinase domain, CheY-like receiver domain and a C-terminal HPt domain. RpfG is a response regulator with a CheY-like receiver domain attached to an HD-GYP domain that acts in degradation of the second messenger cyclic di-GMP (Barber et al. 1997; Ryan et al. 2006). DSF from *X. campestris* has been characterised as the unsaturated fatty acid *cis*-11-methyl-dodecenoic acid (Wang et al. 2004). An ethyl acetate extract from *X. citri* supernatant is active in phenotypic complementation of an *X. campestris rpfF* strain, suggesting a similar structure for DSF in *X. citri*. The *X. fastidiosa* DSF molecule was tentatively identified as 12-methyl-tetradecanoic acid (Colnaghi Simionato et al. 2007).

The current model for DSF signal transduction in *Xanthomonas* is that perception of the signal by the membrane-associated domain of RpfC triggers autophosphorylation of the sensor, followed by phosphorelay and phosphotransfer from the HPt domain to the receiver domain of the RpfG regulator. Phosphorylation of the receiver domain of RpfG alters the activity of the protein in cyclic di-GMP degradation, an activity associated with the HD-GYP domain. Perception of DSF thus leads to a reduction in cellular levels of cyclic di-GMP, with consequences for the regulation of diverse cellular processes (Fig. 3).

Although the Rpf proteins from the different xanthomonads have a high degree of amino acid sequence similarity, the regulatory action of these components is diversified between *Xanthomonas* spp. and *X. fastidiosa*. In *X. campestris* and *X. citri*, *rpfF* mutants, which are DSF deficient, have lower expression of virulence-associated functions such as EPS and extracellular enzymes and reduced virulence to plants (Barber et al. 1997; Siciliano et al. 2006). In contrast, in *X. fastidiosa*, an *rpfF* mutant has enhanced expression of genes encoding the production of the extracellular enzymes such as polygalacturonase (*pglA*) and type IV pili, while those encoding a variety of adhesins are reduced (Chatterjee et al. 2008a). Accordingly, DSF-deficient mutants of *X. fastidiosa* showed enhanced virulence but a reduced capacity to colonise the insect vector (Newman et al. 2004; Chatterjee et al. 2008a).

In *X. citri* and *X. campestris*, *rpfC* mutants have similar phenotypes to *rpfF* mutants. Intriguingly, in *X. fastidiosa*, mutation of *rpfC* has an opposite effect on expression of specific traits to mutation of *rpfF*. The *rpfC* mutant shows reduced virulence but enhanced attachment and biofilm

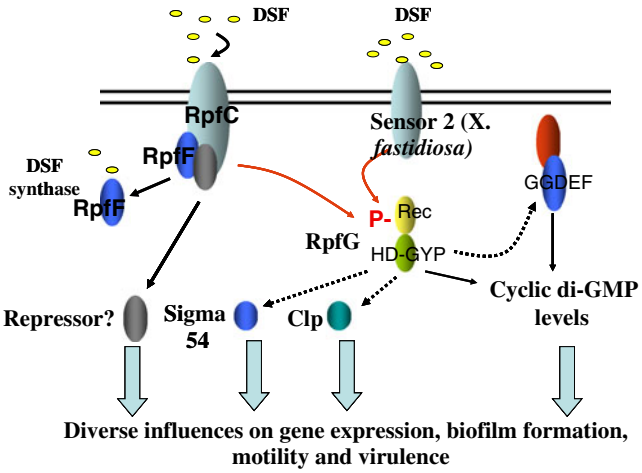

Fig. 3 Signal transduction by the Rpf/DSF signalling system in xanthomonads. RpfF is the enzyme responsible for DSF synthesis whereas RpfC is a sensor kinase responsible for DSF perception. RpfC sequesters RpfF and perhaps other regulators that may be released after conformational changes that occur upon DSF binding, which is believed to be to the RpfC sensory input domain. The consequences are further induction of synthesis of DSF and alteration in expression of particular subsets of genes. Binding of DSF also triggers autophosphorylation of RpfC and phosphorelay to RpfG (*red arrow*), thereby activating cyclic di-GMP degradation by the HD-GYP domain. RpfG may also physically interact with GGDEF domain (diguanylate cyclase) proteins to influence cyclic di-GMP concentrations and with other regulators, modulating their activity in transcription (*dotted lines*). In *Xylella fastidiosa*, a second sensor may phosphorylate RpfG in response to DSF. Although shown as a membrane-bound protein, this sensor may equally be located in the cytoplasm. In *X. campestris*, RpfG influences expression of Clp by an unknown mechanism, leading to activation of a downstream signalling cascade involving other transcriptional regulators. It is not known whether this occurs in *Xylella fastidiosa*

formation, which is correlated with an elevated expression of genes encoding the adhesins HxfA, HxfB and FimA. The *rpfC* mutant can colonise the insect vector (unlike the *rpfF* mutant), but it is not transmitted to the host plant. In contrast to these divergent effects on expression of adhesin genes, mutation of *rpfF* and *rpfC* has similar effects on the expression of genes encoding other virulence determinants; expression of *tolC*, which encodes a protein involved in type I secretion and *pglA* is increased in both *rpfF* and *rpfC* mutants (Chatterjee et al. 2008b).

The occurrence of further regulatory complexity is evident from studies of an *rpfCrpfF* double mutant of *X. fastidiosa*. This strain, which has similar virulence to that of the wild type (and greater virulence than that of the *rpfC* mutant), shows less expression of *tolC* and *pglA* than either of the single mutants. Chatterjee et al. (2008b) have proposed that the decreased expression of the *tolC* and *pglA* in the *rpfCrpfF* double mutant is due to the action of a repressor which is normally sequestered by protein–protein interaction with either RpfC or RpfF. Since DSF has a positive effect on regulation of adhesin genes even in an *rpfC* mutant, Chatterjee and colleagues further propose the existence of two distinct pathways for DSF perception in *X. fastidiosa* (Chatterjee et al. 2008bl; Dow 2008). In the first pathway, in which RpfC acts as the sensor, DSF negatively regulates expression of *rpfF*, *tolC* and *pglA* independently of RpfG and activates genes involved in biofilm formation through RpfG. In the second pathway, a separate (perhaps intracellular) sensor acts in conjunction with RpfG to positively regulate expression of genes linked to biofilm formation (Fig. 3).

RpfC also acts in negative regulation of DSF synthesis. In *Xanthomonas* spp. and in *X. fastidiosa*, mutation of *rpfC* gene results in elevated production of DSF (Chatterjee et al. 2008b). In *X. fastidiosa*, the expression of *rpfF* is increased in the *rpfC* mutant (Chatterjee et al. 2008b) whereas in the *rpfC* mutant of *X. campestris*, the expression of *rpfF* is not changed (Slater et al. 2000). The negative effects of RpfC on DSF synthesis does not require the phosphorelay and may be exerted by sequestration of RpfF by binding to the CheY-like receiver domain of RpfC (He and Zhang 2008). RpfC is thus proposed to have dual signalling functions involving either phosphorelay (for example in the regulation of extracellular enzyme synthesis in *X. citri*) or protein–protein interaction (such as in regulation of DSF synthesis in *X. citri* and *X. fastidiosa* or in modulation of the action of the putative repressor in *X. fastidiosa*).

Diversification in cyclic di-GMP signalling in *X. citri* and *X. fastidiosa*

As outlined above, DSF signal transduction is linked to alteration in the levels of the second messenger cyclic di-GMP through the HD-GYP domain protein RpfG (Fouhy et al. 2006). Two other protein domains, GGDEF and EAL, are also involved in cyclic di-GMP synthesis or degradation; the GGDEF domain is a cyclic di-GMP synthase whereas EAL and HD-GYP are phosphodiesterases, implicated in cyclic di-GMP degradation (D'Argenio and Miller 2004; Dow et al. 2006; Ryan et al. 2006). Many of the proteins with these domains have additional sensory domains, suggesting that their activities are responsive to environmental cues. The complement of proteins with a role in cyclic di-GMP turnover is very different in *X. citri* and *X. fastidiosa*; *X. citri* has 34 proteins with GGDEF, EAL and HD-GYP domains whereas *X. fastidiosa* has 5. Only one of these proteins (RpfG) is common to both bacteria. Cyclic di-GMP exerts an action on multiple regulatory targets in bacteria and it is conceivable that these targets are different in *X. citri* and *X. fastidiosa*. This may in part explain differences in the role of DSF signalling in the two bacteria. Furthermore, yeast two-hybrid analysis

indicates that the HD-GYP domain of RpfG from *X. citri* physically interacts with certain GGDEF domain proteins and also with other regulators such as sigma 54 and NtrBC (Andrade et al. 2006). Although the significance of these interactions to regulation in response to DSF signal transduction is currently unclear, differences in the interactions in *X. citri* and *X. fastidiosa* could also contribute to the different regulatory outputs from a signalling system whose most upstream components are conserved.

Biofilm development and virulence

X. citri and *X. fastidiosa* can develop biofilms, structures in which the bacteria are held together in an extracellular matrix and usually attached to a surface. Biofilm formation is an important factor for pathogenicity (de Souza et al. 2004; Rigano et al. 2007). In *X. fastidiosa*, the mature biofilm morphology in cultures grown on glass cover slips was analysed by light transmission microscopy from 3 to 30 days of growth (de Souza et al. 2004). During the first 5 days, the bacteria became adhered to the glass surface. This phase was followed by formation of small compact aggregates at 15 days. Later, at 30 days, a complete mature biofilm was observed (de Souza et al. 2004). Comparative gene expression between bacteria from this mature biofilm or those grown planktonically for 30 days was examined using DNA microarrays harbouring about 2,200 ORFs from the genome of *X. fastidiosa* strain 9a5c (de Souza et al. 2004). Genes relating to adhesion and adaptation were upregulated in bacteria in the biofilm. These genes included *pilA*, *fimA* and *pilC*, which are involved in the synthesis of pili that have an established role in initial attachment and biofilm formation. Other upregulated genes included those with roles in resistance to antimicrobial agents, the general secretion pathway, synthesis of osmoregulated periplasmic glucans and structural genes for cellulase and proteases (de Souza et al. 2004). In contrast, genes involved in exopolysaccharide (EPS) biosynthesis or in cell–cell signalling (*rpf* genes) were not differentially expressed between biofilm and planktonic cells. This is perhaps surprising, since the *rpf*/DSF system has a role in controlling adhesion in *X. fastidiosa* (Chatterjee et al. 2008b) and EPS is necessary for biofilm formation in *Xanthomonas* spp. (Dow et al. 2000, 2003; Rigano et al. 2007; Torres et al. 2007). As we have seen, the Rpf/DSF system is linked to cyclic di-GMP as a second messenger, and in many bacteria, elevated levels of cyclic di-GMP promote biofilm formation. However, this nucleotide can exert a regulatory influence and act at the posttranslational level, for example, in allosteric activation of polysaccharide synthesis, so that transcriptional profiling alone may not explain all phenotypic variations between biofilm and planktonic cells.

Studies of biofilm in *X. citri* have been performed on static cultures grown in minimal medium containing glucose in chambered covered slides. Biofilms were observed by confocal laser scanning microscopy of bacteria expressing fluorescent proteins (Rigano et al. 2007). After 4 to 5 days under these growth conditions, the wild-type *X. citri* forms a structured biofilm in which the bacteria contact the glass surface through one cell pole and are predominantly attached to each other through lateral interactions to form compact three-dimensional structures. Neither the *rpfF* mutant, which does not produce DSF, nor the *rpfC* mutant, which is a DSF over-producer produces a biofilm with this architecture. Mutants that cannot make EPS are also defective in biofilm formation (Rigano et al. 2007).

The role of bacterial attachment and biofilm formation on colonisation and during canker development was studied in lemon leaves using wild-type and EPS-deficient (biofilm-defective) bacteria expressing fluorescent proteins (Rigano et al. 2007). The findings indicated that the formation of biofilms is important in the epiphytic survival of *X. citri* prior to development of canker disease.

Concluding remarks

Although comparative genomics has revealed differences in the gene complement amongst all three major bacterial pathogens of citrus, it is still unclear how these differences are related to the different lifestyles of the organisms, tissue specificity, adaptation to different environments and interactions with insect vectors. Nevertheless, knowledge of the genome sequences has been extremely useful in identifying candidate genes with a role in virulence in *X. fastidiosa* and *X. citri* and in developing improved detection methods for all three organisms. The determination of the genome sequence of *Ca.* Liberibacter *asiaticus* and the recent report of cultivation of the organism offer the prospect that systems for genetic manipulation can be developed to allow functional genomic analysis. The information from such studies should suggest new strategies for disease control. This may take the form of inhibition of key virulence activities. For example, interference with DSF mediated cell–cell signalling offers a potential route to restrict *X. citri* or *X. fastidiosa* infection. Both signal degradation and signal overproduction may have the desired outcome on disease progression, which could be achieved by the use of epiphytic or xylem-colonising bacteria that degrade the signal, or conversely by genetic manipulation of these

organisms or of the host plant to express RpfF and hence elevate DSF levels. Although the use of chemicals to target the biochemical activity of key virulence factors has been effective in the therapy of human bacterial diseases, satisfactory chemical control of bacterial diseases of plants has not been achieved historically, except for those bacteria that need insect vectors where insecticides may disrupt the disease cycle. In general, the most effective management measure of plant disease, apart from use of good agronomic practices, is the deployment of genetic resistance to pathogens. The identification of new bacterial virulence factors and mechanisms should permit the development of assays to reveal natural genetic variation in plants or to guide the manipulation of targets in the laboratory.

Acknowledgements MRM, APC and AAV are Career Investigators of Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Argentina and are supported by Agencia Nacional de Promoción Científica y Tecnológica. JMD is supported by a Principal Investigator Award 07/IN.1/B955 from the Science Foundation of Ireland. AMdoA received a fellowship from CNPq. We thank Luciano Kishi for his help with the Venn diagram.

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