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Recent Progress in Understanding the Roles of DSF-regulated Virulence Factors in *Xanthomonas campestris* Pathogenicity

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ABSTRACT

Xanthomonas campestris pathovar *campestris* (*Xcc*) is the causal agent of black rot disease of cruciferous plants. A cell-cell signaling system encoded by genes within the *rpf* cluster is required for the full virulence of this plant pathogen. This system has been implicated in regulation of production of extracellular enzymes, cyclic glucan and the exopolysaccharide xanthan and in the regulation of biofilm formation in *Xcc*. Cell-cell communication is mediated by the diffusible signal factor (DSF), an unsaturated fatty acid whose synthesis requires RpfF and RpfB. Here we review current progress on our understanding of DSF signal transduction and of the roles of xanthan, cyclic glucan and biofilm development in the interaction of *Xcc* with plants. Recent observations have shown that the perception of the DSF signal requires the sensor kinase RpfC and is linked to the degradation of the intracellular second messenger cyclic di-GMP by the HD-GYP domain regulator RpfG. The mechanisms by which cyclic di-GMP exerts its regulatory influence on xanthan, cyclic glucan and biofilm formation remain obscure however. It is now established that DSF signaling has to be finely balanced for the formation of structured biofilms in static cultures in minimal medium and for virulence to plants. New roles for xanthan and cyclic glucan in disease through suppression of plant immune responses have been uncovered. Xanthan induces susceptibility to *Xcc* in *Nicotiana benthamiana* and *Arabidopsis thaliana* by suppressing callose deposition. Unlike xanthan, which acts only locally, the effects of cyclic glucan on plant defense suppression and callose deposition occur in a systemic fashion. These advances contribute to the increased understanding of the molecular basis of bacterial disease, which is a major aim in the post-genomic era in plant-bacterial interactions.

Keywords: *Arabidopsis thaliana*, glucan, *Nicotiana benthamiana*, plant defense xanthan, *Xanthomonas*
Abbreviations: CLSM, confocal laser-scanning microscopy; **EPS**, exopolysaccharide; **GlcUA**, glucuronic acid

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INTRODUCTION

Xanthomonas campestris pv. *campestris* (*Xcc*) is the causal agent of black rot, an economically important disease which affects cruciferous crops worldwide (Onsando 1992). As with many phytopathogenic bacteria, *Xcc* produces a range

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of factors that contribute to the ability of the bacterium to parasitise the host (Daniels *et al*. 1994). Among these are extracellular enzymes capable of degrading plant cell wall polymers and other plant components and extracellular polysaccharide (EPS). The EPS produced by *Xcc* is called xanthan, and is a polymer composed of repeating pentasaccharide units with the structure mannose-1,4-β-glucuronic acid-1,2-β-mannose-1,3-α-cellobiose (Jansson *et al*. 1975). *Xcc* also produces a neutral cyclic glucan containing 16 glucose residues. Cyclic glucan can be found both in a periplasmic location within bacterial cells (Talaga *et al*. 1996) and in culture supernatant (Amemura and Cabrera-Crespo 1986; York 1995).

In *Xcc* the production of extracellular enzymes, cyclic glucan and xanthan are subject to co-ordinate positive regulation by a cluster of genes, the *rpf* cluster (for regulation of pathogenicity factors) (Tang *et al*. 1991). Mutations in *rpf* genes lead to reduced virulence in host plants. Several of the *rpf* genes encode components of a cell-cell communication system mediated via a small diffusible molecule, which has been called DSF (for diffusible signal factor) (Barber *et al*. 1997; Slater *et al*. 2000). DSF has been characterised as the unsaturated fatty acid *cis*-11-methyl-dodecenoic acid (Wang *et al*. 2004). In addition to control of synthesis of extracellular enzymes, cyclic glucan and xanthan (Vojnov *et al*. 2001b), DSF signaling controls biofilm dispersal in rich medium (Dow *et al*. 2003).

Recent work has shed more light on both the mechanisms of DSF signal transduction and the role of DSF-controlled processes in promoting bacterial disease. Here we review these findings, specifically addressing the role of cyclic di-GMP as a second messenger in DSF signaling, the action of xanthan and extracellular cyclic glucan in suppression of plant defense responses, and the fine balance of DSF synthesis that is required for biofilm formation in minimal medium and for optimal virulence to plants. In this way we hope to highlight the newly discovered strategies that *Xcc* uses to cause disease in the host plant.

CELL-CELL SIGNALING IN *XANTHOMONAS*

The *rpf* gene cluster and cell-cell signaling in *Xanthomonas*

As introduced above, the synthesis of several virulence factors involved in *Xcc* pathogenicity is controlled by the products of the *rpf* genes (Tang *et al*. 1991). This cluster comprises nine genes, *rpf A* to *I* and is located within a 21.9 kb region of the *Xcc* chromosome. The left part of this region contains six contiguous *rpf* genes with the gene order *rpf ABFCHG*. Mutations in any of these genes lead to co-ordinate down regulation of the synthesis of all the extracellular enzymes, cyclic glucan and EPS (Barber *et al*. 1997; Vojnov *et al*. 2001). The *rpfBFGHC* genes encode components of the DSF cell-cell signaling system. RpfF and RpfB direct the production of the DSF signal molecule (Barber *et al*. 1997), which has been characterized as the unsaturated fatty acid *cis*-11-methyl-dodecenoic acid (**Fig. 1A**) (Wang *et al*. 2004). The synthesis of DSF is completely dependent on RpfF, which has a certain amino acid sequence similarity to enoyl-CoA hydratases, but is only partially dependent on RpfB, which is a long chain fatty acyl CoA ligase. The *rpfB* and *rpfF* genes are co-transcribed from a promoter upstream of *rpfB*, although *rpfF* also has its own promoter (Slater *et al*. 2000). The *rpfF* mutants can be phenotypically corrected for the production of extracellular enzymes, cyclic glucan and EPS by the exogenous addition of DSF or by growth on plates in proximity to a wild type strain (Barber *et al*. 1997; Vojnov *et al*. 2001b).

Perception of the DSF signal requires the two-component system comprising RpfC and RpfG, which are encoded within the *rpfGHC* operon, which is contiguous with *rpfF* but convergently transcribed (Slater *et al*. 2000). RpfC is a complex sensor kinase with a predicted membrane-associated sensory input domain as well as histidine kinase, CheY-like receiver (REC) and C-terminal histidine phosphotransfer (HPt) domains. RpfG is a novel regulator with a CheY-like receiver (REC) domain and an HD-GYP domain. Although the amino acid sequence of RpfH resembles that of the sensory input domain of RpfC, no role for RpfH in

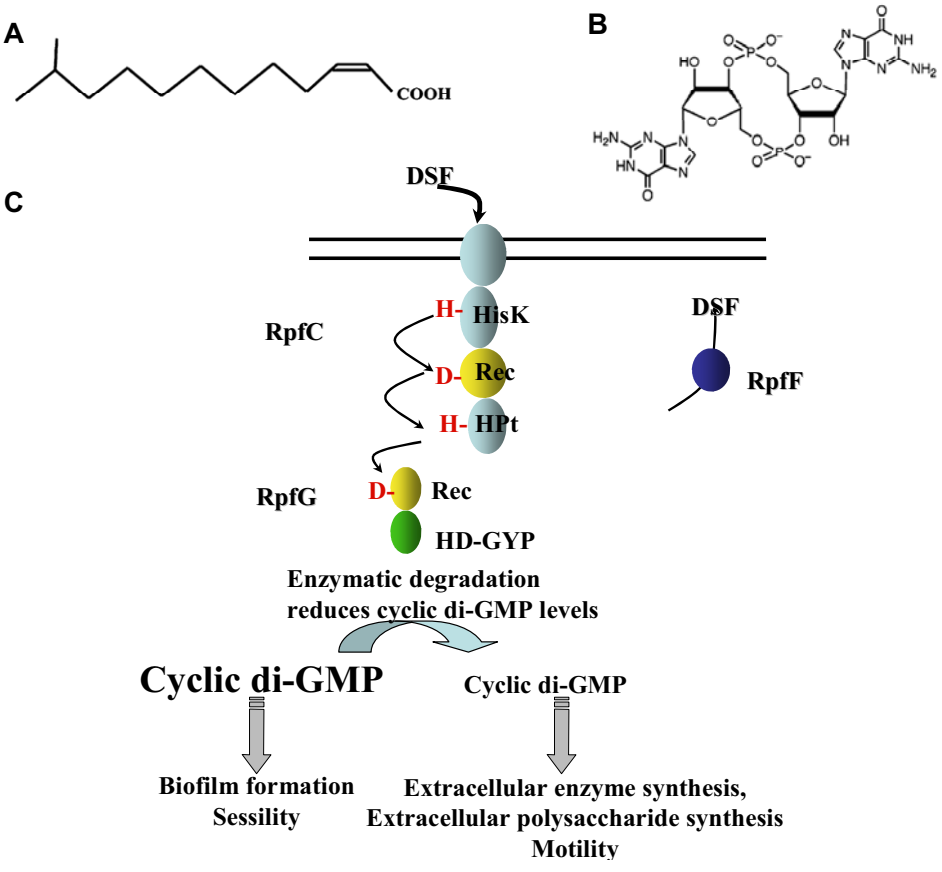

Fig. 1 (**A**) Structure of DSF. (**B**) Structure of cyclic di-GMP. (**B**) Model for regulation of virulence factor production by *rpf*/DSF system. The synthesis of the DSF signal requires RpfF and is partially dependent on RpfB. DSF perception and signal transduction involves the complex sensor RpfC and HD-GYP domain regulator RpfG, which is a cyclic di-GMP phosphodiesterase. By analogy with a number of related sensor proteins, signal transduction may involve autophosphorylation of RpfC in response to ligand (DSF) binding, followed by phosphorelay and phosphotransfer to the cognate regulator, in this case RpfG (indicated by arrows). Phosphorylation of RpfG leads to its activation as a cyclic di-GMP phosphodiesterase, an activity associated with the HD-GYP domain. The consequent alterations in the level of cyclic di-GMP affect the synthesis of virulence factors such as extracellular enzymes, biofilm dispersal and motility by as yet unknown mechanisms. RpfC may recognise additional environmental cues. KEY to domains: Boxes: putative transmembrane helices; REC: CheY-like two-component receiver domain; HPt: histidine phosphotransfer; HisK: histidine kinase.

DSF signaling or regulation of extracellular enzyme or xanthan synthesis is yet apparent and *rpfH* mutants retain full virulence (Slater *et al*. 2000; Dow *et al*. 2003). In addition to positive regulation of virulence factor synthesis, RpfC acts to negatively regulate DSF synthesis, a function that does not involve RpfG (Slater *et al*. 2000).

The remaining *rpf* genes (*rpfA*, *rpfD*, *rpfE* and *rpfI*) have no apparent involvement in the DSF-dependent production of *Xcc* virulent factors and have minor regulatory roles (Barber *et al*. 1997; Wilson *et al*. 1998; Dow *et al*. 2000). The function of some of these Rpf proteins has been described or can be predicted from their amino acid sequence. Accordingly, RpfA is an aconitase that may play a role in iron homeostasis (Wilson *et al*. 1998), and RpfD has a LytTR DNA-binding domain (IPR007492) (Nikolskaya and Galperin 2002), suggesting a role in transcriptional activation. RpfE and RpfI are conserved hypothetical proteins (Dow *et al*. 2000).

Dual signaling functions of RpfC involve either phosphorelay or receiver domain-protein interactions

As outlined above, RpfC acts to positively regulate virulence factor synthesis in response to DSF but to negatively regulate the synthesis of DSF itself. Recent work has shown that these dual signaling functions are achieved by different mechanism (He *et al*. 2006a, 2006b). Work on sensor kinases with related domain structure (such as BvgS of *Bordetella* spp. and and ArcB of *Escherichia coli*) has implicated autophosphorylation as a consequence of signal perception followed by phosphorelay via REC and HPt domains to the cognate regulator as the mechanism of signal transduction. By analogy it was proposed that RpfC worked in the same fashion (Slater *et al*. 2000; He *et al*. 2006a, 2006b). Mutational analysis of the three conserved amino acid residues of RpfC implicated in phosphorelay (H198 in the histidine kinase domain, D512 in the REC domain and H657 in the HPt domain) showed that they are essential for activation of the production of extracellular enzymes and xanthan, but not for repression of DSF biosynthesis. Domain deletion analyses revealed that the REC domain of RpfC alone was sufficient to repress DSF overproduction in an *rpfC* mutant. This may involve a physical interaction between the REC domain and RpfF, the enzyme involved in DSF biosynthesis, as suggested by co-immunoprecipitation and far western blot analyses.

These data support a model in which RpfC modulates the different functions of virulence factor synthesis and DSF synthesis by utilization of a conserved phosphorelay system (**Fig. 1C**) and a novel domain-specific protein-protein interaction mechanism, respectively. In this model sequestration of RpfF by RpfC renders it inactive in DSF synthesis. Structural changes in RpfC, perhaps as a result of DSF binding and auto-phosphorylation, allow release of RpfF, which is then active in DSF synthesis. In this view, perception of DSF would be auto-inductive on its synthesis, but this would not involve changes in expression of the *rpfF* gene. This is consonant with the finding that transcript levels of *rpfF* are only modestly elevated over wild type in an *rpfC* mutant, whereas DSF levels are considerably higher (Slater *et al*. 2000). Although the model is consistent with the available data, it cannot be excluded that DSF synthesis is additionally regulated at other levels, perhaps by the supply of the substrates for RpfB/RpfF or post-transcriptional control of the expression of RpfF and RpfB proteins.

DSF signal transduction and cyclic di-GMP degradation

As outlined above, perception of the DSF signal is thought to activate the autophosphorylation of RpfC and result in phosphorelay and phosphotransfer to the REC domain of the RpfG regulatory protein. RpfG is an unusual two-component regulator in that it has an HD-GYP domain attached to the REC domain, rather than a DNA binding domain as seen in the majority of such regulators (Slater *et al*. 2000). The HD-GYP domain is a subset of the HD superfamily of metal dependent phosphohydrolases (Galperin *et al*. 1999, 2001). Bioinformatic studies have suggested a role for the HD-GYP domain in the degradation of the bacterial second messenger cyclic di-GMP (Galperin *et al*. 1999, 2001). Recent experimental studies have shown that the HD-GYP domain is indeed a novel cyclic di-GMP phosphodiesterase (Ryan *et al*. 2006a), thus implicating cyclic di-GMP in DSF signal transduction.

Cyclic di-GMP (bis-(3-5)-cyclic di-guanosine monophosphate) is an almost ubiquitous second messenger in bacteria that was first described as an allosteric activator of cellulose synthase (Ross *et al*. 1990), but it is now known to regulate a range of functions, including biofilm formation, motility, developmental transitions, virulence factor synthesis and the virulence of human and animal pathogens (D'Argenio and Miller 2004; Jenal 2004; Paul *et al*. 2004; Romling and Amikam 2006). The structure of cyclic di-GMP is shown in **Fig. 1B**. Two protein domains, GGDEF (IPR000160) and EAL (IPR001633), are implicated in the synthesis and degradation respectively of cyclic di-GMP (Scarpari *et al*. 2003; Paul *et al*. 2004; Christen *et al*. 2005; Ryjenkov *et al*. 2005). Synthesis of cyclic di-GMP by the GGDEF domain occurs from GTP, whereas EAL domains are phosphodiesterases that convert cyclic di-GMP into the linear nucleotide pGpG. GGDEF and EAL domains are widely distributed in bacteria including plant pathogens. The majority of proteins containing these domains have additional signaling domains, suggesting that their activities are responsive to different environmental cues (Galperin *et al*. 2001; Romling *et al*. 2005; Romling and Amikam 2006). In general high cellular levels of cyclic di-GMP promote biofilm formation and sessile growth, whereas low levels promote virulence factor synthesis and motility (Galperin *et al*. 2001; Romling *et al*. 2005; Romling and Amikam 2006).

Indirect evidence for the role of RpfG in cyclic di-GMP turnover has come from experiments where GGDEF and EAL domain proteins have been ectopically expressed in *Xcc* wild type and *rpfG* mutant. Expression of genes encoding EAL domain proteins in the *Xcc rpfG* mutant restores extracellular enzymes. In contrast expression of genes encoding a GGDEF domain protein in wild type *X. campestris* gives a phenocopy of the *rpfG* mutant (Ryan *et al*. 2006). These indirect observations are consistent with a role for the HD-GYP domain in cyclic di-GMP hydrolysis. This conclusion was supported by biochemical studies that demonstrate that the isolated domain can hydrolyse cyclic di-GMP to GMP via the linear intermediate pGpG (Ryan *et al*. 2006). Mutation of the HD residues comprising the presumed catalytic diad of the HD-GYP domain abolishes both the regulatory activity and enzymatic activity against cyclic di-GMP. Further support for a role of cyclic di-GMP in DSF signal transduction has come from experiments in which the RpfC/RpfG two-component system has been re-constructed in *Pseudomonas aeruginosa* and shown to confer responsiveness to exogenously added DSF as seen through effects on swarming motility (Ryan *et al*. 2006a). It has been proposed that phosphorylation of RpfG leads to its activation in cyclic di-GMP hydrolysis (Fouhy *et al*. 2006; **Fig. 1C**) although this has not been directly demonstrated.

The link of DSF signal perception to cyclic di-GMP degradation raises the related issues of whether other cyclic di-GMP signaling systems in *Xcc* regulate the same functions as RpfG and how such a system with many potential players is functionally organized. A comprehensive mutational analysis of the role of all 37 proteins with HD-GYP, GGDEF and EAL domain proteins in regulation of extracellular enzyme synthesis and motility in *Xcc* has been recently reported (Ryan *et al*. 2007). A number of proteins in addition to RpfG act to regulate extracellular enzyme synthesis, although different proteins have significant roles under different growth conditions. RpfG is the only protein to have an influence under all growth conditions tested and

loss of RpfG has the biggest effect on extracellular enzyme synthesis. These findings are consistent with the concept of a signaling network that includes the RpfC/RpfG system and that responds to and integrates information from a number of cues including the DSF cell-cell signal. Conversely other signaling elements in *Xcc* regulate motility but have no effect on extracellular enzyme production. This is consistent with the concept of localised action of certain elements in cyclic di-GMP signaling.

The DSF 'regulon' and signal transduction beyond RpfG

The full extent of the DSF 'regulon' has been examined by transcriptome profiling (He *et al*. 2006b). It is evident that DSF signaling regulates a number of functions in addition to extracellular enzymes and extracellular polysaccharide with a potential contribution to bacterial virulence; these include resistance to oxidative and other stresses, iron assimilation and motility (He *et al*. 2006b). Several recent studies have addressed the molecular details of the DSF signal transduction beyond RpfG, which are not well understood. The mechanism(s) by which cyclic di-GMP exerts its regulatory influences in *Xcc* is unknown. Work in other bacteria has implicated PilZ, a cyclic di-GMP binding domain, as an adaptor in the regulatory action of cyclic di-GMP (Amikam and Galperin 2006; Ryjenkov *et al*. 2006). There are four PilZ domain-containing proteins in *Xcc*, whose regulatory roles have yet to be examined. The HD-GYP domain of RpfG from the related pathogen *X. axonopodis* pv. *citri* has been shown by yeast two-hybrid analysis to interact with a subset of GGDEF domain proteins (Andrade *et al*. 2006). Although this may suggest an action of RpfG in modulating the activity of specific cyclic di-GMP generating systems, the biological relevance of such interactions remains to be investigated.

The DSF/*rpf* system has also been shown to activate transcription of the gene encoding the cyclic-AMP receptor-like protein Clp (He *et al*. 2007). In *Xcc*, Clp regulates many functions including the expression of genes for extracellular enzymes and EPS synthesis, and those for the regulators Zur and FhrR. In turn Zur regulates genes for functions such as iron uptake, the TCA cycle, multidrug resistance and detoxification, whereas FhrR regulates expression of genes for flagellar synthesis and type III secretion (He *et al*. 2007). The available evidence indicates that not all of the regulatory effects of RpfG are exerted through the action of Clp. For example Clp is not apparently involved in regulation of biofilm dynamics in *Xcc* (He *et al*. 2007).

ROLE OF XANTHAN IN THE *XANTHOMONAS*-PLANT INTERACTIONS

Xanthan as a virulence factor

The synthesis of xanthan is certainly required for *Xcc* pathogenicity. Mutation of genes involved in the synthesis of xanthan reduces bacterial survival within *Brassica campestris* leaves. When inoculated into the mesophyllic tissue, populations of such mutants are two to three orders of magnitude lower in comparison with those of the wild type (Newman *et al*. 1994). In the field, bacteria normally enter intact leaves through the hydathodes at the leaf margins and then multiply and spread within xylem vessels. Vein blackening, the first visible symptom (and which gives the disease its name), and disorganization of the vascular tissues soon follows the obstruction of vessels. The synthesis of xanthan is required not only for the early stages of hydathode colonisation (Hugouvieux *et al*. 1998), but is also thought to contribute to the xylem obstruction (Leigh and Coplin 1992). The role and function of bacterial EPS has been, and still is, a matter of some discussion. Xanthan may contribute to resistance to desiccation and the action of reactive oxygen compounds produced by challenged plants Xanthan is also known to contribute to biofilm formation by *Xcc* (Dow *et al*. 2003) and this is discussed in the context of new findings on biofilm formation in a later part of the review on biofilms. In the following sections we briefly describe the structure of xanthan and its synthesis before going on to discuss the relatively recent discovery of its role in suppression of plant defenses.

Chemical structure of xanthan and synthesis

The chemical structure of xanthan consists of a 'cellulose' backbone of β-1,4 linked glucose residues with a side chain comprising the trisaccharide mannose-β-1,4-glucuronic acid-β-1,2-mannose-α-1,3 attached to every other glucose. Xanthan is also substituted with pyruvate and acetate moieties. A complete structure is represented in **Fig. 2A**. The synthesis of xanthan involves the assembly of the pentasaccharide repeating unit while linked to a polyprenol through a diphosphate bridge. Subsequently, the repeating unit is polymerized and the polymer secreted outside the cell body (Ielpi *et al*. 1993). The genes that encode for the enzymes involved in the transfer of the sugars and of the non-glycosidic substituents are located in a cluster which comprises 12 predicted open-reading frames, *gumB–gumM* (Thorne *et al*. 1987; Katzen *et al*. 1996, 1998; Vojnov *et al*. 2002). Transcriptional analysis has shown that the *gum* genes are mainly expressed as an operon from a promoter upstream of the first gene, *gumB* (Katzen *et al*. 1996) (**Fig. 2B**).

Xanthan production *in planta* and its regulation

To investigate the timing of xanthan production within plants, a reporter construct was created by fusion of the region of the *gum* gene cluster that is immediately upstream of *gumB* gene with the coding sequence for β-glucuronidase of *Escherichia coli* (*gus A*). The expression of the *gumgusA* reporter was maximal during stationary phase of growth, of bacteria grown in liquid cultures and when bacterial populations had reached maximal levels in plant mesophyll tissue (Vojnov *et al*. 2001). The level of expression of *gumgusA in planta* was reduced in an *rpfF* mutant compared to the wild type, as is also seen in liquid cultures. This suggests a positive regulation of xanthan synthesis by *rpfF* (and hence by DSF signaling) occurs within the plant host (Vojnov *et al*. 2001).

A similar pattern of expression was also observed for the *eps* operon of *Ralstonia solanacearum* (formerly known as *Pseudomonas solanacearum* and *Burkholderia solanacearum*) which directs the biosynthesis of EPS I, the complex exopolysaccharide of this tomato pathogen (Kang *et al*. 1999). The *eps* operon is only activated at later stages of infection. The results strongly suggest that both *Xcc* and *R. solanacearum* produce large amount of EPS only at later phases of disease.

There are several possible reasons why bacteria would not wish to produce large amounts of EPS during early phases of pathogenesis. A limited EPS production may allow adherence of bacterial colonies to plant cell surfaces promoting the establishment of biofilms or microcolonies, and may allow the proper functioning of type III secretion systems encoded by the *hrp* gene clusters (for hypersensitive resistance and pathogenicity), which are critical for the establishment infection. Copious production of EPS early in pathogenesis might interfere with these processes. Conversely high production of EPS at later stages of disease in tissues undergoing necrosis might protect the bacteria against various stresses, such as desiccation and damage by reactive oxygen species liberated by as a part of the plant defense response.

Xanthan as suppressor of plant defense

Recent studies have examined the importance of xanthan production and xanthan structure for *Xcc* virulence in *Nicotiana benthamiana* and *Arabidopsis thaliana*. The behaveour of the wild type strain was compared with that of and

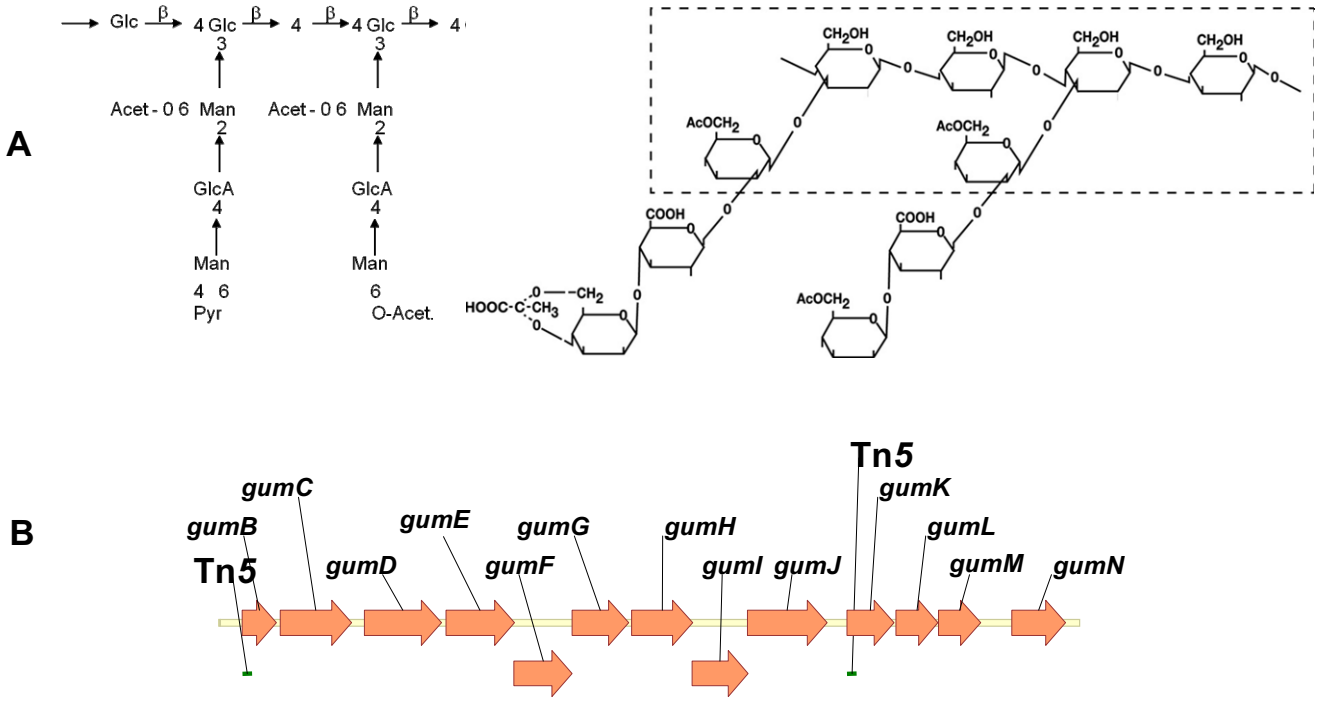

Fig. 2 (**A**) Xanthan structure. Two repeating units are represented to show the different substitutions in the external mannoses. Glc = glucose; GlcA = glucuronic acid; Man = mannose; O-Acet = Acetyl group O linked; Pyr = ketalpyruvate. (**B**) *gum* gene function: *gumB* = polymerization and export; *gumC* = polymerization and export; *gumD* =tranferase I = Glc 1-P transferase; *gumM* = tranferase II = Glctransferase; *gumH* = tranferase III = Man 1 transferase; *gumK* = tranferase IV = GlcA transferase; *gumI* = tranferase V = Man 2 transferase; *gumF* = Acetyl transferase; *gumG* = Acetyl transferase II; *gumL* = Ketal pyruvate transferase.

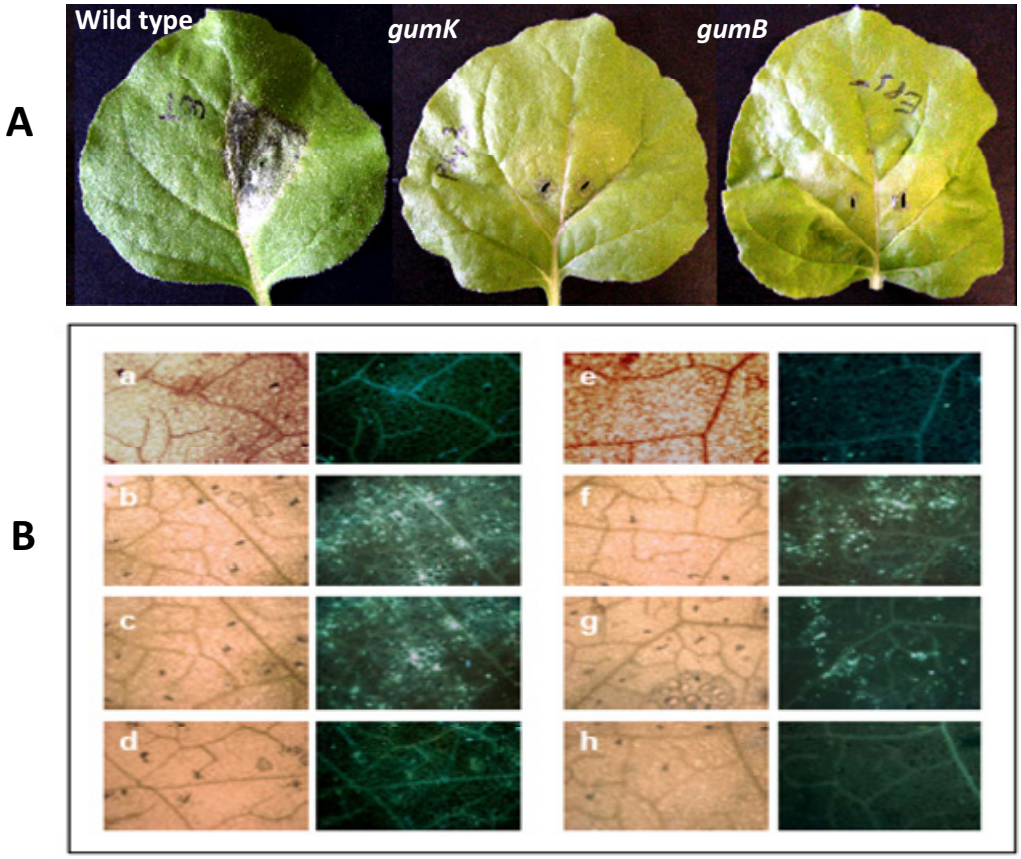

Fig. 3 Xanthan is essential for pathogenicity and suppresses callose deposition. (**A**) Infection of *N. benthamiana* with *Xcc* strains. Symptoms in *N. benthamiana leaves* after inoculation with either wild type *Xcc* strain, *Xcc ndvB* mutant and *gumK* mutant. (**B**) Callose deposition in *N. benthamiana* leaves is associated with resistance and is suppressed by the *Xcc* exopolysaccharide xanthan. *N. benthamiana* leaves were pretreated with water, xanthan, or truncated xanthan and inoculated with strains of *Xcc*. The leaves were then stained for callose deposits 24 h post inoculation (white dots) and observed by light (left, light sections) and fluorescence microscopy (right, dark sections). a: wild type, b: *gumB*, c: *gumB* + polytrisaccharide, d: *gumB* + xanthan, e: water, f: *gumK*, g: *gumK* + polytrisaccharide, h: *gumK* + xanthan.

two mutants carrying Tn*5* transposon insertions in different genes responsible for the xanthan synthesis (**Fig. 2B**). One of these mutants, with an insertion immediately upstream of the *gumB* gene, is unable of produce xanthan (Vojnov *et al*. 1998). The other mutant carries an insertion in *gumK* gene that encodes the glucuronosyl transferase enzyme (**Fig. 2B**). This mutant is unable to transfer glucuronic acid residues to the lipid-linked repeating unit, producing truncated side chains (**Fig. 2A**, dotted line). Nevertheless, the trisaccharide repeating unit is polymerized into a high molecular-weight polysaccharide, with a substituted cellulose-like structure, that is released into the growth medium (Vojnov *et al*. 2002).

The *Xcc gumB* mutant was not pathogenic in either *A. thaliana* or *N. benthamiana* and did not incite disease symp-

toms as seen with the wild type strain (**Fig. 3A**). These studies confirmed previous findings (Newman *et al*. 1994) that showed an essential function of xanthan in the pathogenic interaction of *Xcc* with plants. The relevance of the structure and composition of the polysaccharide was also studied by use of the *gumK* mutant, which produces a truncated xanthan polymer. The *gumK* mutant showed reduced virulence when inoculated into leaves of *A*. *thaliana* and an absence of symptoms when inoculated into *N*. *benthamiana* (Yun *et al*. 2006) (**Fig. 3A**). The reduced capacity in the production of symptoms of *gumK* mutant gave the first indication of the importance of the structure and composition of the EPS in *Xcc* infectivity.

Examination of the plant response to *Xcc* wild type and *gum* mutants has given an insight into a potential role for xanthan in defense suppression during disease. Inoculation of plants with *gumB* or *gumK* mutants leads to an enhanced deposition in the plant cell wall of callose, a beta (1,3) glucan with 1,6 modifications that is associated with increased resistance of plants against some pathogens (Stone BA 1992; Hamiduzzaman *et al*. 2005). This effect is not seen with the wild type. Pre-treatment of plants with xanthan from the wild type, but not the polytrisaccharide produced by *gumK* mutant, is able to suppress this callose deposition (Yun *et al*. 2006) (**Fig. 3B**). These results show a role of xanthan as suppressor of the plant defense and further suggest that the presence of the negatively charged glucuronosyl and ketal-pyruvate residues in the xanthan might be essential for this biological function during bacterial-plant interaction. It has been previously shown that local increase in Ca^{2+} ions can directly activate the callose synthase enzyme and initiate callose formation (Kohle *et al*. 1985). One mechanism by which xanthan could act to suppress cell wall-based plant defense would therefore be binding of extracellular calcium ions, with consequent interference of signal transduction linked to callose synthetase activation. This ability to bind Ca^{2+} will depend on its negative charge, which is conferred by the presence of glucuronosyl residues and through ketal-pyruvate substitution. The loss of negative charge in the EPS from the *gumK* mutant hence renders it inactive in suppression (Yun *et al*. 2006).

ROLE OF β-(1,2) CYCLIC GLUCAN IN *XANTHOMONAS*-PLANT INTERACTIONS

The DSF signaling system regulates the level and cellular distribution of cyclic glucan in *Xcc* by mechanisms that remain obscure. Studies of *rpfF* and *rpfC* mutants show that compared to the wild type they have lower levels of cell-associated glucan and produce only trace levels in the culture supernatants (Vojnov *et al*. 2001). In addition to *rpf* regulation, the levels of cyclic glucan in *Xcc* are subject to osmotic control (Vojnov *et al*. 2001), as has been found for periplasmic glucans in a wide range of bacteria.

Osmoregulated periplasmic glucan families

Four families of osmoregulated periplasmic glucan have been recognised based on the structural features of the polyglucose backbone. The family I glucans, which are found in *Escherichia coli*, and the plant pathogens *Erwinia chrysanthemi* and *Pseudomonas syringae*, are highly branched structures consisting of a backbone of β-1,2-linked glycosyl residues to which the branches are attached by β-1,6-linkages. The plant pathogenic *Agrobacterium* spp. and symbiotic *Rhizobium* and *Sinorhizobium* spp. synthesise family II glucans which have cyclic β-1,2-linked glucose backbones of heterogeneous size. The family III glucans such as those found in *Bradyrhizobium japonicum*, are also cyclic, but differ from those of family II by having β-1,6- and β-1,3 links instead of β-1,2 and by a strict control of ring size. The glucan produced by *Xcc* falls into family IV, a group that also includes the cyclic glucan of *Ralstonia solanacearum*, another important plant pathogen (Breedveld and Miller 1994; Bohin 2000). They all have one α-1,6 linkage with the rest with β-1,2 (Bohin 2000).

The three distinct sets of genes that direct the synthesis of glucans of the families I, II and III have been defined respectively as the *mdo* family, the *ndv* family and a third family with one representative found in *Bradyrhizobium japonicum*. In *Escherichia coli*, two genes forming the *mdoGH* operon are required for glucan synthesis; closely related genes (including *hrpM*), are found in *Pseudomonas syringae*. In *Sinorhizobium meliloti*, two linked genes (*ndvA* and *ndvB*) are required for cyclic glucan synthesis. Closely related genes to *ndvA* and/or *ndvB* are found in *Agrobacterium tumefaciens* (*chvA* and *chvB*), *Sinorhizobium fredii* (*ndvB*) and *Brucella abortus* (*cgs*) (Bohin 2000).

Glucan participation in bacterial-host interactions

Evidence from mutational analysis of those genes involved in the synthesis of glucans suggests a role for this compounds in the interaction of phytopathogenic and symbiotic bacteria with plants as well as bacterial pathogens with animal hosts (Geremia *et al*. 1987; Arellano-Reynoso *et al*. 2005; Rigano *et al*. 2007). In many cases structural genes for glucan synthase were isolated through screens for bacterial mutants with attenuated virulence or loss of pathogenicity, before the precise role of the genes in glucan synthesis was established. Accordingly *chv* mutants of *Agrobacterium tumefaciens* and *hrpM* mutants of *Pseudomonas syringae* do not produce symptoms of disease in normally susceptible plants (Douglas *et al*. 1985; Loubens *et al*. 1993). Similarly, *ndvB* mutants of *Sinorhizobium meliloti* form defective nodules on alfalfa (Geremia *et al*. 1987).

Mutants in glucan synthase genes were first characterised in several model organisms (*E*. *coli*, *P*. *syringae*, *A*. *tumefaciens*, *S*. *meliloti*). The loss of cyclic glucan synthesis in *Sinorhizobium meliloti* through mutation of *ndvB* gives rise to a pleiotropic phenotype where nodulation and motility are both compromised (Geremia *et al*. 1987). Closely related genes from other bacteria producing the same family of periplasmic glucan as *S*. *meliloti* (family II) are able to complement these defects in the *ndvB* mutant of *S*. *meliloti*. Interestingly, genes from *Bradyrhizobium japonicum*, which produces a family III periplasmic glucan, are also able to complement (Bohin 2000).

Genes encoding the glucan synthase in *Xanthomonas*

Xcc produces a neutral cyclic β-1,2-glucan with 16 glucose residues with 15 β-1,2-linked glycosyl residues and one α-1,6-linked residue (Amemura and Cabrera-Crespo 1986; York 1995; Talaga *et al*. 1996) (**Fig. 4A**).

According to their homology with identified sequences in other bacteria, there are three candidate genes for glucan synthase in *Xcc*: (1) *hrpM* with homology to *mdoA* of *E*. *coli*, *Erwinia* and *Pseudomonas*, (2) a gene highly similar to the *ndvB* (nodulation development) and *chvB* (chromosomal virulance) genes of *Rhizobium* and *Agrobacterium* respectively, and (3) *XC_4168*, which has homology to the 3' end of the *ndvB* gene (da Silva *et al*. 2002). As is found in *Rhodobacter sphaeroides*, inactivation of the *Xcc* gene related to *ndvB*, has no consequence in cyclic glucan synthesis in *Xcc* (Cogez *et al*. 2002). In contrast, the disruption of *XC_4168* compromises the production of the extracellular cyclic β-(1,2)-glucan in *Xcc* (Rigano *et al*. 2007), thus identifying it as the synthase responsible for cyclic glucan synthesis in *Xcc*. The construction of a defined mutant with a disruption of this gene permitted the assessment of the role of cyclic glucan in the interaction of *Xcc* with plants (Rigano *et al*. 2007). The function of the candidate genes homologous to *ndvB* and *hrpM* in *Xcc* remains unclear.

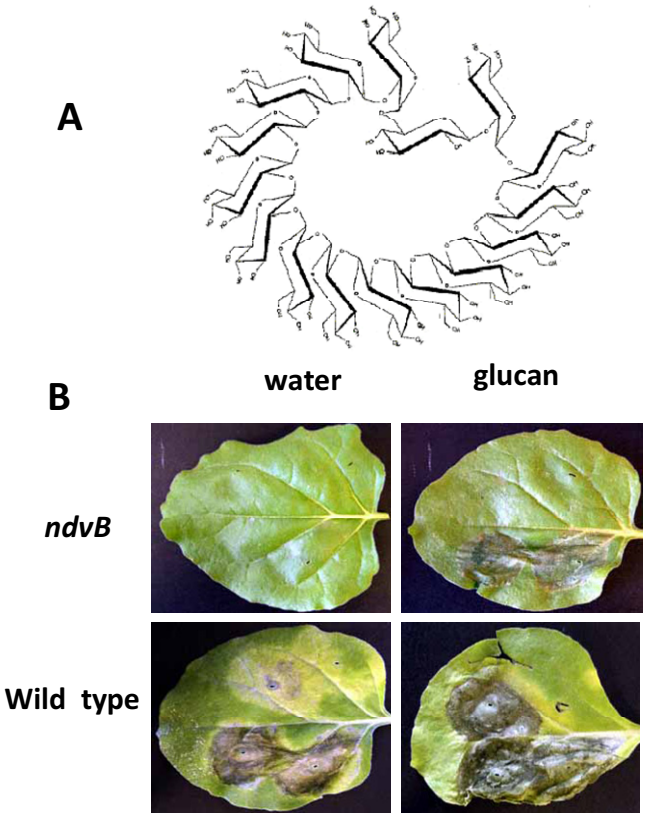

Fig. 4 Cyclic glucan structure and importance in *X. campestris*-*N. benthamiana* interaction. (**A**) Cyclic β-1,2-glucan of *X. campestris* containing 16 glucose residues with 15 β-1,2-linked glycosyl residues and one α-1,6-linked residue. B. *Xcc* cyclic glucan suppresses disease resistance in *N. benthamiana*. Leaves of 4-week-old plants were preinfiltrated with glucan or water and after 24 h a 10^7 cfu/mL suspension of either *Xcc* wild-type strain 8004 or *ndvB* mutant strain were inoculated.

Glucan as suppressor of plant defense during *Xanthomonas* infection

The effects of inactivation of the *Xcc XC_4168 ndvB* gene on disease development were evaluated in the model plants *N. benthamiana* and *A. thaliana* (Rigano *et al*. 2007). The behaviour of the *Xcc ndvB* mutant *in planta* was compared to that of the wild type by analyzing the production of symptoms and bacterial growth kinetics after bacterial infiltration. In addition, the defense-related responses of the synthesis of callose and the induction of the gene encoding the defense-related protein PR1 were monitored (Rigano *et al*. 2007).

The *ndvB* mutant shows an absence of disease symptoms after leaf inoculation (**Fig. 4B**, left upper picture), which is associated with attenuated growth and lower final population size compared to the wild type (**Fig. 4B**, left lower picture). In addition, clear differences are observed between wild type and *ndvB* mutant in terms of callose deposition and the accumulation of transcripts for *PR-1*. After wild type *Xcc* inoculation, *PR-1* transcripts are barely detectable at 12 h and reach a maximum accumulation at 24 h. In contrast, in response to the *ndvB* strain, accumulation of *PR-1* transcripts reach high levels at 12 h, and this state is maintained until 24h after inoculation. Leaves challenged with the *Xcc ndvB* mutant strain show considerably enhanced callose deposition compared to leaves inoculated with the wild type strain. Taken together, the lower bacterial numbers attained, the more rapid induction of *PR-1* and alteration in the plant cell wall suggest that the host is exhibiting a resistance response to the *ndvB* strain. This suggests that *Xcc* cyclic β-(1,2)-glucan may have a role in inducing host susceptibility through suppression of plant defense. These conclusions are supported by experiments which show that pre-treatment of leaves with cyclic (1,2) glucan suppresses *PR1* induction and callose deposition by the *ndvB* mutant and restores virulence (Rigano *et al*. 2007). In correlation with *PR1* and callose synthesis suppressions, pre-treatment with glucan complemented the *ndvB* mutant symptoms and increased the *Xcc* wild type aggressiveness (**Fig. 4B**, right upper and right lower pictures, respectively).

The translocation of cyclic glucan in plants and the induction of systemic susceptibility to *Xcc*

To further understand the role of glucan in the suppression of defense responses, *N. benthamiana* leaves were pre-infiltrated with purified glucan followed by bacterial inoculation of the pre-treated (local) or non-pre-treated (distal) leaves with *Xcc* wild type and the *ndvB* mutant strain. Evaluation of disease symptoms, bacterial growth, callose deposition and *PR1* expression indicated cyclic glucan acted to suppress plant defenses not only locally but in a systemic fashion (Rigano *et al*. 2007). These latter findings rise the immediate question of what signal is translocated through the plant to induce disease susceptibility in the distal leaves? One possibility is that glucan interaction with the plant cell triggers production of a second molecular signal for systemic susceptibility that is translocated. Another possibility is that the glucan itself is the translocated signal. This issue was partially resolved by the demonstration that enzymatically-synthesized ^{14}C-glucan could be translocated in an intact form from inoculated leaves to those that were not inoculated. Although the ability of the cyclic glucan to exert a systemic effect on disease suppression is correlated with the systemic movement of the molecule, currently it is not possible to formally exclude the involvement of a second translocated signal. Some bacterial toxins have been shown to be able to spread in plant in the absence of the producing pathogens (Mitchell and Bieleski 1977). Furthermore, coronatine, a toxin produced by *Pseudomonas syringae*, also induces systemic susceptibility in plants (Cui *et al*. 2005). It is still unknown however if systemic effects induced by coronatine require translocation of the molecule itself or occur via local activation of the jasmonic acid pathway.

BIOFILM FORMATION AND VIRULENCE IN *XANTHOMONAS*

DSF-mediated signaling is required for the dispersal/formation of biofilm in *Xcc*

Biofilms have been defined as matrix-enclosed bacterial populations that are adherent to each other and/or to surfaces or interfaces. As with animal and human pathogens, the ability of bacterial plant pathogens to form and detach from biofilms may be considerable implications for the completion of the disease cycle. The DSF cell-cell signaling system is known to regulate the dispersal of biofilms formed by *Xcc* in shaken rich nutrient medium. Under these growth conditions, *rpfG*, *rpfC* and *rpfF* mutants form matrix-enclosed aggregates whereas the wild-type strain does not (Dow *et al*. 2003). The formation of these aggregates requires xanthan (Dow *et al*. 2003). Addition of exogenous DSF to culture medium causes aggregate dispersal in *rpfF* but not in other *rpf* mutants. These experiments in rich nutrient medium suggest an effect of DSF on biofilm dispersal but with no influence on biofilm formation (Dow *et al*. 2003).

The effects of DSF on *Xcc* biofilm formation appear to depend greatly on the growth conditions. Recent work has reported an examination of *Xcc* growing in static minimal medium cultures in chambered covered slides (Russo *et al*. 2006), using CLSM imaging of bacteria expressing fluorescent proteins. Under these conditions, the wild type forms a structured biofilm, although *rpfF* (DSF non-producer) and *rpfC* (DSF over-producer) strains do not (Torres *et al*. 2007). In the formation of a typical *Xcc* biofilm, the bacteria contacted the glass surface via the lateral cell surface and they

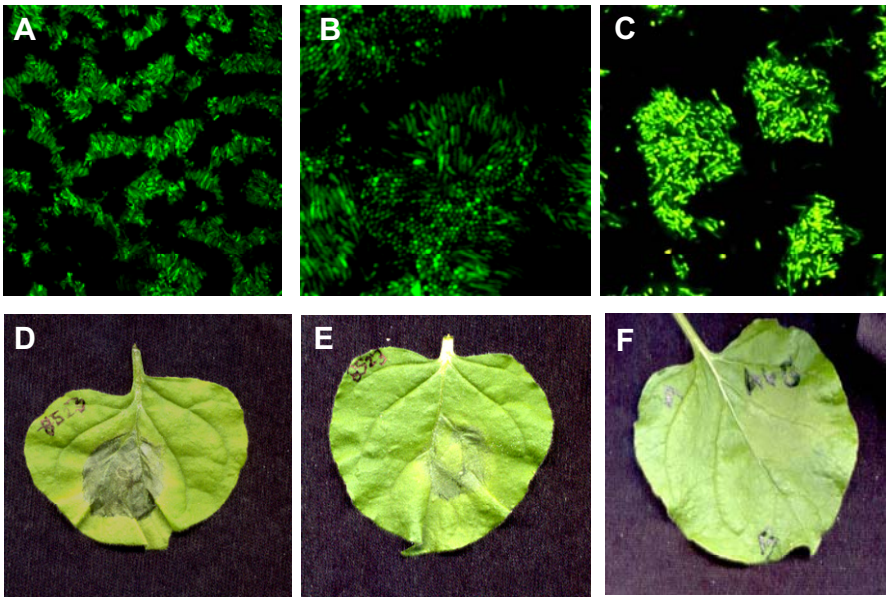

Fig. 5 Biofilm formation and virulence in *Xanthomonas*. (**A** and **B**) Biofilms formed after 4 days by wild type-gfp strain of *Xcc* in minimal medium culture. (**C**) images acquired from mixed cultures of *rpfF* and *gumB* carrying the GFP-expressing plasmid and the EYFP-expressing plasmid, respectively. (**D-F**) Symptom production in *Nicotiana benthamiana* by *Xcc* strains wild type (**D**), *rpfF* (**E**) and *rpfC* (**F**).

predominantly attached to each other through lateral interactions forming compact three-dimensional aggregates structure (**Fig. 5A, 5B**). The formation of these structures requires xanthan and does not occur in a *gumB* mutant (Torres *et al*. 2007).

DSF synthesis has to be fine-tuned for biofilm formation and virulence

Evidences for a fine-tune regulation of DSF necessary for biofilm development and virulence has come from experiments using mixed cultures of *Xcc* strains (Torres *et al*. 2007). The xanthan-deficient *gumB* mutant and the *rpfF* mutant are unable to produce a structured biofilm in single culture in static minimal medium, however in mixed cultures of the two strains a structured biofilm develops. The interpretation is that extracellular complementation has occurred, in which DSF produced by the *gumB* mutant restores xanthan production by the *rpfF* mutant to allow a structured biofilm to develop (**Fig. 5C**). Structured biofilms formed in this way comprise a mixture of the two bacteria as demonstrated by the use of a GFP-labelled *rpfF* mutant and an EYFP-labelled *gumB* mutant (**Fig. 5C**). This indicates that the extracellular complementation is reciprocal, as xanthan produced by the *rpfF* mutant is allowing the incorporation of the *gumB* mutant into the structured biofilm. In contrast to these findings, substitution of the *gumB* mutant with the DSF overproducing *rpfC* mutant under the same conditions did not allow development of a structured biofilm. These observations suggest complementation is possible only when an appropriate concentration of DSF was present; it is not the case that any level beyond a particular threshold will suffice.

The biological implication of altering DSF levels on the interaction between *Xcc* and plants have also been evaluated by co-inoculations of *N. benthamiana* with mixed cultures of different mutants. Almost no symptoms are observed in *N. benthamiana* when the *rpf* mutants or *gumB* strain are inoculated singly, demonstrating the importance of the xanthan and other virulence factors regulated by *rpf*/DSF system in *Xcc* infection (**Fig. 5E, 5F**). However leaves of *N. benthamiana* co-inoculated with *gumB* and *rpfF* strains show similar disease symptoms to those produced by the wild type strain of *Xcc*. In contrast, co-inoculation of *rpfC* (DSF overproducer) and *rpfF* (DSF non-producer) strains did not allow disease symptoms to occur (Torres *et al*. 2007). Taken together these recent findings suggest that DSF signaling is finely balanced during both biofilm formation and virulence.

CONCLUDING REMARKS

It is clear that the synthesis of virulence factors by pathogenic bacteria is tightly regulated, can occur as a response to different environmental cues, and may alter during disease progression. The factors we have discussed here are regulated by cell-cell signaling through DSF, a molecule whose levels in the immediate bacterial environment will be responsive to a number of factors including the number of bacteria producing the signal and the volume in which they may be confined. DSF signaling may be particularly important for example for bacteria confined within the xylem elements, where they may be at a relatively high cell density but perhaps less so for bacteria on leaf surfaces. The DSF system does not appear to have any significant regulatory overlap with the *hrp* (for hypersensitive reaction and pathogenesis) regulon. It may be that the two systems operate sequentially during disease, with *hrp* genes important for establishment of an infection and DSF-regulated factors for the progression of the disease. DSF-regulated factors may also have roles in other phases of the *Xcc* disease cycle, such as bacterial survival in soil on dead plant parts and epiphytic growth.

The appreciation that DSF signal transduction is linked to alteration in the levels of the second messenger cyclic di-GMP is important since it opens the possibility that synthesis of virulence factors may be under the influence of regulatory networks of cyclic di-GMP signaling systems which respond to a range of environmental cues. By extension, this could suggest that DSF signaling may be relatively unimportant under certain environmental conditions. Other unrelated regulatory systems also impinge on the synthesis of virulence factors such as xanthan, which is costly in terms of metabolic energy.

Many questions arise from the results of the recent investigations that we review here. Although sequestration of Ca^{2+} is a plausible mechanism for the suppression of plant defenses by xanthan, does cyclic glucan act in the same way? Is their any interplay between cyclic glucan and type III secreted effectors, some of which also act to suppress basal resistance responses? Is there a plant receptor for cyclic glucan? An understanding of the action of cyclic glucan in plants may suggest new strategies for plant protection. Furthermore, the demonstration that DSF synthesis is tuned for optimal virulence and biofilm formation in minimal medium suggests that such a fine balance might therefore be readily disrupted. This may have substantial consequences for development of measures for the control of diseases caused by *Xcc* and perhaps other *Xanthomonas* spp.

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