Controlled synthesis of the DSF cell–cell signal is required for biofilm formation and virulence in *Xanthomonas campestris*

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Summary

Virulence of the black rot pathogen *Xanthomonas campestris* pv. *campestris* (*Xcc*) is regulated by cell–cell signalling involving the diffusible signal factor DSF. Synthesis and perception of DSF require products of genes within the *rpf* cluster (for regulation of pathogenicity factors). RpfF directs DSF synthesis whereas RpfC and RpfG are involved in DSF perception. Here we have examined the role of the *rpf*/DSF system in biofilm formation in minimal medium using confocal laser-scanning microscopy of GFP-labelled bacteria. Wild-type *Xcc* formed microcolonies that developed into a structured biofilm. In contrast, an *rpfF* mutant (DSF-minus) and an *rpfC* mutant (DSF overproducer) formed only unstructured arrangements of bacteria. A *gumB* mutant, defective in xanthan biosynthesis, was also unable to develop the typical wild-type biofilm. Mixed cultures of *gumB* and *rpfF* mutants formed a typical biofilm *in vitro*. In contrast, in mixed cultures the *rpfC* mutant prevented the formation of the structured biofilm by the wild-type and did not restore wild-type biofilm phenotypes to *gumB* or *rpfF* mutants. These effects on structured biofilm formation were correlated with growth and disease development by *Xcc* strains in *Nicotiana benthamiana* leaves. These findings suggest that DSF signalling is finely balanced during both biofilm formation and virulence.

Introduction

Xanthomonas campestris pv. *campestris* (*Xcc*) is the causal agent of black rot disease which affects cruciferous crops worldwide (Onsando, 1992). As with many phytopathogenic bacteria, *Xcc* produces a range of factors that contribute to the ability of the bacterium to parasitize the host (Dow and Daniels, 1994). Among these are extracellular enzymes capable of degrading plant cell components and an extracellular polysaccharide (EPS) called xanthan. These factors may play a number of roles during disease. Extracellular enzymes may be required to overcome plant defence responses, to allow bacteria to move into uncolonized plant tissues and to mobilize plant polymers for nutritional purposes. Xanthan induces susceptibility to *Xcc* in *Nicotiana benthamiana* and *Arabidopsis thaliana* by suppressing basal defences such as callose deposition (Yun *et al.*, 2006), has a role in biofilm formation (Dow *et al.*, 2003) and may have further roles in protecting bacteria from stresses of desiccation and host-elaborated defences.

In *Xcc* the production of extracellular enzymes and EPS is subject to coordinate positive regulation by a cluster of genes, the *rpf* cluster (for regulation of pathogenicity factors) (Tang *et al.*, 1991; Dow and Daniels, 1994;

Barber *et al.*, 1997; Slater *et al.*, 2000). Mutations in *rpf* genes lead to a reduced virulence in host plants. Several of the *rpf* genes mediate regulation via a small diffusible molecule named DSF (for <u>d</u>iffusible <u>s</u>ignal <u>f</u>actor) (Barber *et al.*, 1997). DSF has recently been structurally characterized as *cis*-11-methyl-2-dodecenoic acid (Wang *et al.*, 2004). The synthesis of DSF is directed by RpfB and RpfF and DSF perception and signal transduction is mediated by the RpfC/RpfG two-component system, which is encoded by the *rpfGHC* operon (Slater *et al.*, 2000). Mutation of *rpfC*, which encodes the sensor component, leads to overproduction of DSF and to lower levels of EPS and extracellular enzymes (Tang *et al.*, 1991; Slater *et al.*, 2000). The addition of DSF can phenotypically restore *rpfF* but not *rpfC* mutants to wild type for production of extracellular enzymes and EPS (Barber *et al.*, 1997). Regulation of EPS biosynthesis by DSF occurs at least in part at the level of transcription of the *gum* operon, which encodes the sugar transferases required for EPS biosynthesis (Vojnov *et al.*, 1998; 2001; 2002). There is circumstantial evidence for the operation of the DSF regulatory system *in planta* (Vojnov *et al.*, 2001).

More recent work has implicated the DSF signalling system in the regulation of biofilm dispersal in *Xcc* (Dow *et al.*, 2003). When grown in a rich medium containing glucose, *rpfF*, *rpfG*, *rpfC* and *rpfGHC* mutants form aggregates in which the bacteria are held together in a polymeric matrix. The integrity of this matrix is dependent on the synthesis of xanthan (Dow *et al.*, 2003). Aggregates formed by *rpfF* mutants disperse upon addition of DSF, but those formed by other *rpf* mutants do not. As the wild type grows in a dispersed planktonic form under these conditions, it was concluded that the role of DSF was in induction of biofilm dispersal in *Xcc*.

Growth conditions are known to influence many aspects of bacterial behaviour including the formation of biofilms. The work in this article was prompted by the observation that when grown in minimal medium (conditions that may more closely mimic those found in planta), the wild-type *Xcc* formed a structured biofilm on glass slides. Here we have examined the role of DSF signalling in biofilm formation in minimal medium with confocal laser-scanning microscopy (CLSM) of GFP-tagged *Xcc* strains, used both singly and in pairwise combinations. We provide evidence that biofilm formation requires tight control of the level of the DSF, with both DSF overproduction and non-production adversely affecting the formation of a structured biofilm. Furthermore, examination of the effects of inoculation of combinations of strains in the model plant *N. benthamiana* provided evidence for the action of DSF within plants and suggested that balanced DSF levels were required for optimal virulence.
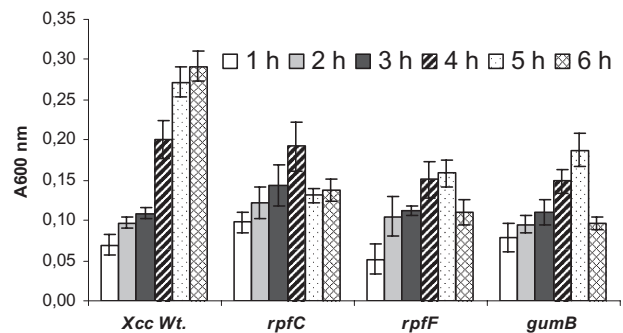

Fig. 1. Attachment of *Xcc* to polystyrene is reduced by mutations affecting the *rpf*/DSF signalling system and xanthan synthesis. Bacteria attached to the surfaces of wells in microtitre plates were stained with crystal violet, and the absorbance at 600 nm was measured during 6 h. The strains used were 8004 (wild type), 8523 (*rpfF*), 8557 (*rpfC*) and 8397 (*gumB*). Data are presented as the mean absorbance values plus standard errors for four replicate wells from a representative experiment.

Results

The development of a structured Xcc *biofilm is under the control of the* rpf/*DSF system*

The ability of *Xcc* to form biofilms was examined in the minimal Y medium (see *Experimental procedures*). In preliminary experiments, bacterial adhesion to polystyrene 96-well plates was analysed by crystal violet staining. Interestingly, the *rpfF*, *rpfC* and xanthan-deficient *gumB* mutants showed significantly less adherence than the wild-type strain 8004 after 6 h of growth in Y-minimal medium (Fig. 1). This is in contrast to effects seen in rich L medium, where levels of attachment of *rpfG*, *rpfGHC* and *gum* mutants were higher than the wild type (Crossman and Dow, 2004).

The characteristics of the *Xcc* biofilm formed *in vitro* in minimal medium were analysed by CLSM over a 4-day time-course experiment of static cultures in chambered cover slides (Russo *et al.*, 2006). In the formation of a typical *Xcc* biofilm, the bacteria contacted the glass surface via the lateral cell surface and also predominantly attached to each other through lateral interactions forming microcolonies (Fig. 2, day 2). This phase was followed by the formation of compact aggregates of bacteria with a characteristic three-dimensional structure separated by extensive water spaces (Fig. 2, day 4). A *z*-projection of the *x–y* stacks (optical sections) showed a mushroom-type biofilm structures (Fig. 2). Bacteria in these structures were mostly interacting laterally (Fig. 2, day 4).

With the *rpfF* mutant (DSF-minus), microcolonies were seen after 2 days, but these did not develop into a structured biofilm, so that after 4 days only unstructured layers of bacteria were observed. With the *rpfC* mutant (DSF overproducer), although the bacteria showed some aggregation at day 2, only unstructured layers of bacteria
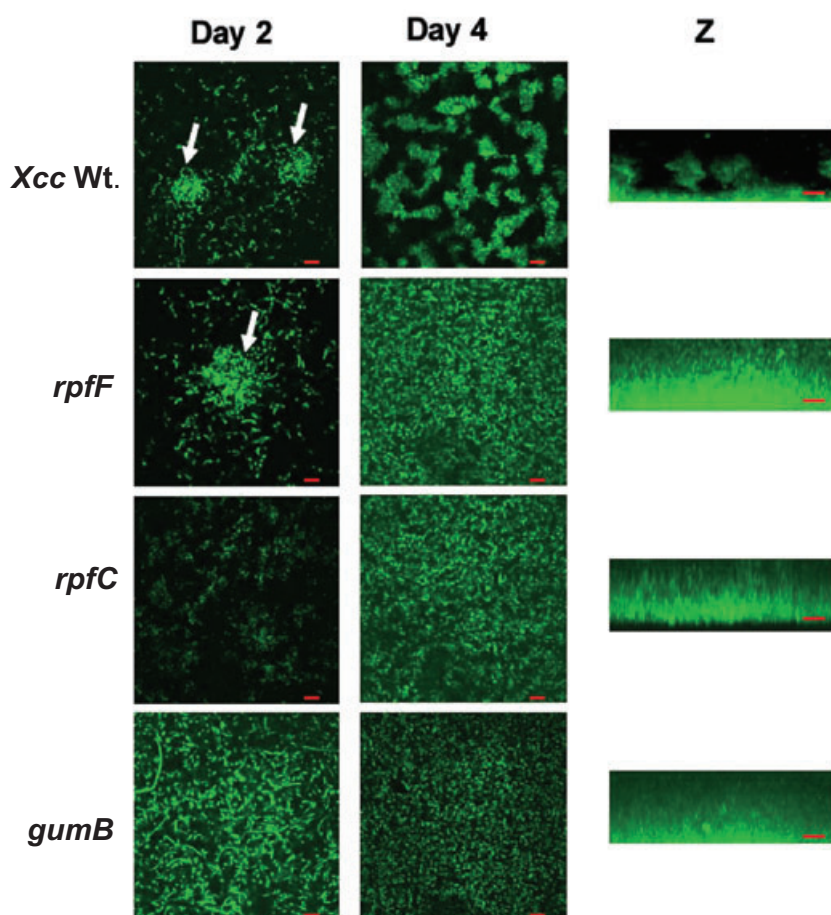
Fig. 2. Development of biofilms in strains of *Xcc*. Strains 8004 (wild type), 8523 (*rpfF*), 8557 (*rpfC*) and 8397 (*gumB*) containing the GFP-expressing plasmid pRU1319 were grown for 4 days in chambered cover slides and observed daily by CLSM. The panels (left to right) show day 2, day 4 in a layer at 6, 4 µm from the base glass and the day 4 *z*-axis projected images. Arrows indicate microcolonies. Scale bars = 5 µm.

were observed at day 4 (Fig. 2). The relative levels of DSF of the different strains, which were predicted from behaviour in other media, were confirmed experimentally for growth in minimal medium in static culture (see Fig. S1). Overall these results showed that DSF-mediated signalling is required for the formation of a structured biofilm in minimal medium.

Role of xanthan in the formation of the structured biofilm in minimal medium

To evaluate the importance of xanthan in the formation of the structured biofilm, the behaviour of the *gumB* mutant grown in Y medium was analysed by CLSM. We observed that the *gumB* mutant (strain 8397) was severely affected in the microcolony formation and did not form more complex structures (Fig. 2). After 4 days, no evident biofilm architecture was observed on the base of the chamber (Fig. 2, *z*-stage). The *gum* cluster of genes cloned in pIZD261-15 restored normal levels of EPS and a typical structured biofilm to *gumB* strain 8397 (not shown). These observations confirmed that xanthan synthesis in *Xcc* is crucial for the development of the struc-

tured biofilm in Y medium. Measurement of the relative levels of xanthan in the static cultures showed that after 2 days of growth, both *rpfF* and *rpfC* mutants had significantly lower levels than the wild type (see Fig. S2), consistent with previous observations of the role of the *rpf*/ DSF system in regulating synthesis of this polysaccharide (Tang *et al.*, 1991; Slater *et al.*, 2000; Vojnov *et al.*, 2001).

Extracellular complementation of biofilm-defective phenotypes in mixed bacterial cultures

Our CLSM analysis indicated that mutations in *rpfF* and *gumB* genes resulted in the absence of a typical and structured biofilm. To confirm that this phenotype is due to the contribution of extracellular DSF and secreted xanthan, mixed cultures of *gumB* and *rpfF* strains, both GFP-labelled, were analysed over a time-course experiment by CLSM. The mixed culture of the *gumB* and the *rpfF* mutants was able to induce cell clustering and to develop a structured biofilm (Fig. 3D). These results suggested that reciprocal complementation had taken place, where the lack of DSF in the *rpfF* mutant had been restored by DSF produced by the *gumB* mutant and the xanthan produced
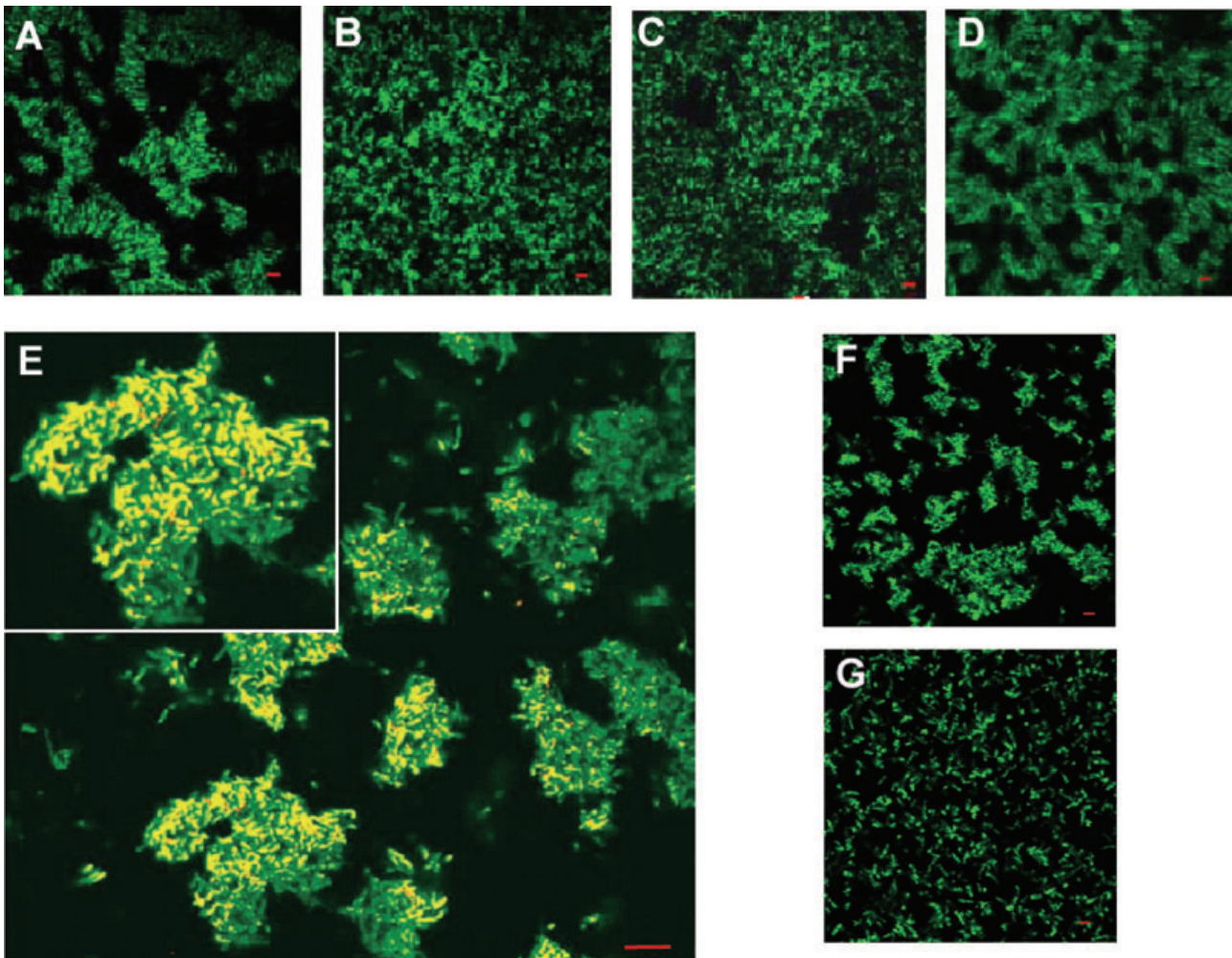
Fig. 3. Biofilms formed by mixed *Xcc* cultures.
A–E. Biofilms formed after 4 days by mixed cultures are shown for the following. Wild type-gfp + *rpfF*gfp (A), wild type-gfp + *rpfC*gfp (B), *rpfF*gfp + *rpfC*gfp (C) and *rpfF*gfp + *gumB*gfp (D). All the inoculations were at a 1:1 ratio of the two strains. (E) shows dual-colour confocal images acquired from mixed cultures of *rpf* and *gumB* carrying the GFP-expressing plasmid (pRU1319) or the EYFP-expressing plasmid (pMP4518) respectively.
F and G. Biofilms formed after 4 days by the *rpfF*gfp strain supplemented with DSF extracted from 4-day static culture of the wild type (F) or *rpfF* mutant (G). Scale bars = 5 μm.

by the *rpfF* mutant was substituting for the lack of xanthan in the *gumB* mutant. Structured biofilms formed in this way comprised a mixture of the two bacteria as demonstrated by the use of a GFP-labelled *rpfF* mutant and an EYFP-labelled *gumB* mutant (Fig. 3E).

To investigate whether regulated production of DSF was needed for biofilm development, the wild-type strain 8004 was co-inoculated with the *rpfC* mutant (Fig. 3). At a 1:1 ratio the ability of 8004 to form the wild-type structure was abolished (Fig. 3B). At a 1:4 ratio of *rpfC* mutant to wild-type strain, only slightly modifications of the biofilm were observed (not shown). Similar results were obtained in mixed cultures when both *rpfC* and wild-type strains were GFP-labelled or when only the wild-type strain was GFP labelled (not shown). This eliminated the possibility

that the *rpfC* mutant simply out-competes the wild-type strain for attachment, thereby always giving an *rpfC*-like pattern. Co-inoculation of the *rpfC* mutant with the *rpfF* mutant strain 8523 (DSF-defective) in a 1:1 ratio did not restore the formation of the typical wild-type structure (Fig. 3C). In contrast to the effects caused by the *rpfC* mutant, the *rpfF* mutant did not alter the wild-type 8004 biofilm when the two strains were mixed (Fig. 3A).

Taken together with the results of reciprocal complementation of *rpfF* and *gumB* mutants, these findings suggest that the amount of DSF produced has to be tightly controlled for the development of the biofilm and increased levels of DSF interfere with this process. These conclusions were supported by the results of experiments in which exogenous DSF was added to cultures. Addition
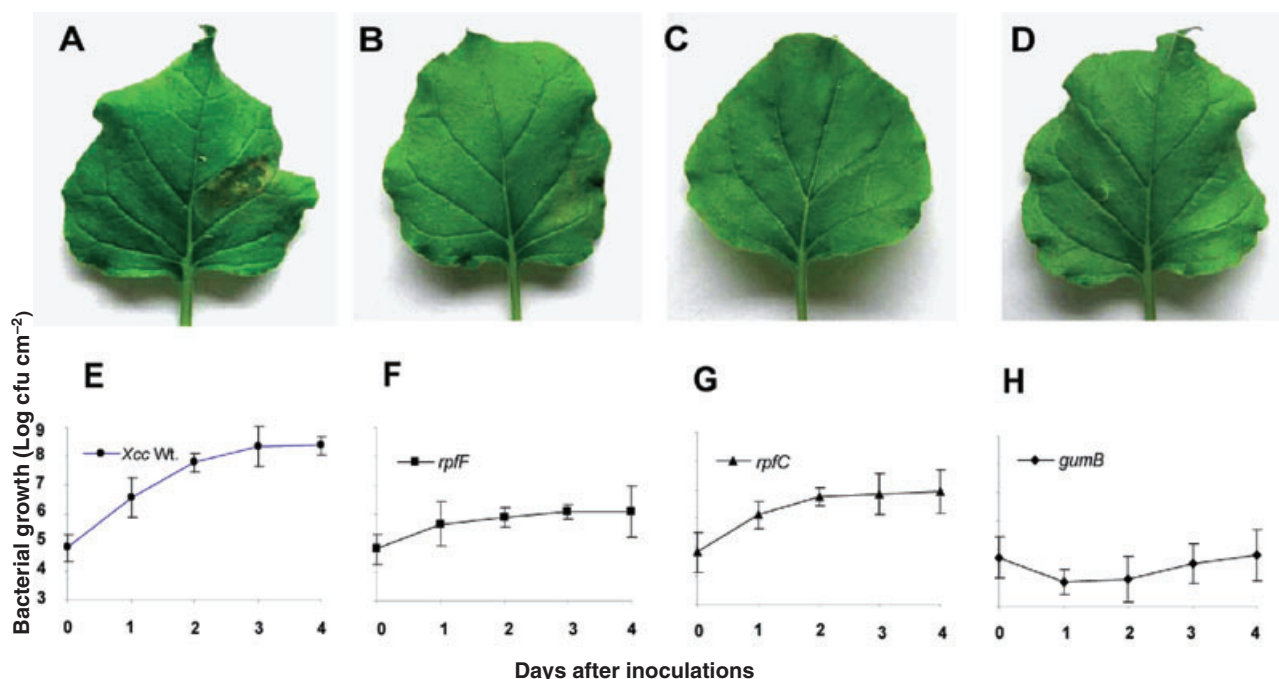
Fig. 4. Growth and symptom production by *Xcc* strains in *Nicotiana benthamiana*. Symptoms and growth were determined after inoculation into *N. benthamiana* leaves of *Xcc* strains 8004 (wild type) (A and E), 8523 (*rpfF*) (B and F), 8557(*rpfC*) (C and G) and 8397 (*gumB*) (D and H).

of DSF extracted from a 4-day culture of the wild type to a culture of the *rpfF*/GFP mutant allowed the production of a structured biofilm at 4 days (Fig. 3F). Extracts from cultures of the *rpF* mutant (DSF-minus) by contrast had no effect on biofilm structure (Fig. 3G). Furthermore, addition of DSF extracted from the *rpfC* mutant (DSF overproducer) to cultures of the wild-type strain 8004 culture inhibited structured biofilm development (not shown).

Phenotypic characterization of in planta *behaviour of* rpf *mutants and* in vivo *complementation studies*

On the basis of the above findings, we aimed to investigate the possible biological implication of altering DSF levels on the interaction between *Xcc* and plants. Our model pathosystem was the interaction of *Xcc* with *N. benthamiana* (Yun *et al.*, 2006). *Nicotiana benthamiana* has become a useful model plant, primarily because it shows an unusual susceptibility to a variety of pathogens. In initial experiments, we analysed the virulence phenotype of the *rpf* mutants, the *gumB* mutant and wild-type strain 8004 in *N. benthamiana* (Fig. 4). Each strain was inoculated in a leaf of *N. benthamiana* and symptoms and bacterial growth were monitored (see *Experimental procedures*). In contrast to the wild type, all mutant strains produced almost no symptoms in *N. benthamiana* (Fig. 4A–D). With the wild-type strain the number of colony-forming units (cfu) recovered from leaf disks cut from the inoculated area

increased more than three orders of magnitude over 4 days of infection (Fig. 4E). In contrast, both the *rpfF* and *rpfC* strains showed significantly less growth after the same infection period (Fig. 4F and G). As previously reported (Yun *et al.*, 2006), the xanthan-defective *gumB* mutant was completely asymptomatic on the *N. benthamiana* leaves (Fig. 4D) and was severely compromised in growth in the plant tissue (Fig. 4H).

To examine the role of DSF cell–cell signalling during *Xcc* pathogenesis, co-inoculations of *N. benthamiana* with mixed cultures of different mutants were performed. Although leaves of *N. benthamiana* inoculated with *gumB* and *rpfF* separately showed no symptoms associated with limited bacterial growth (Fig. 4), when the two strains were inoculated together a different outcome was seen. In this case, the growth of both the *gumB* and the *rpfF* in *N. benthamiana* increased significantly (Fig. 4G) and symptoms induced by the mixed culture were similar to those induced by the wild type (Fig. 5C). These observations strongly suggested that reciprocal complementation was occurring. That is, DSF produced by the *gumB* mutant was complementing the DSF deficiency of *rpfF* and that xanthan secreted by the *rpfF* strain complemented the defect in the *gumB* strain. From these findings we inferred that DSF cell–cell signalling occurred *in planta*.

To examine whether regulated production of DSF was required for virulence, *N. benthamiana* leaves were inoculated with the wild-type 8004 and the *rpfC* mutant in a 1:1
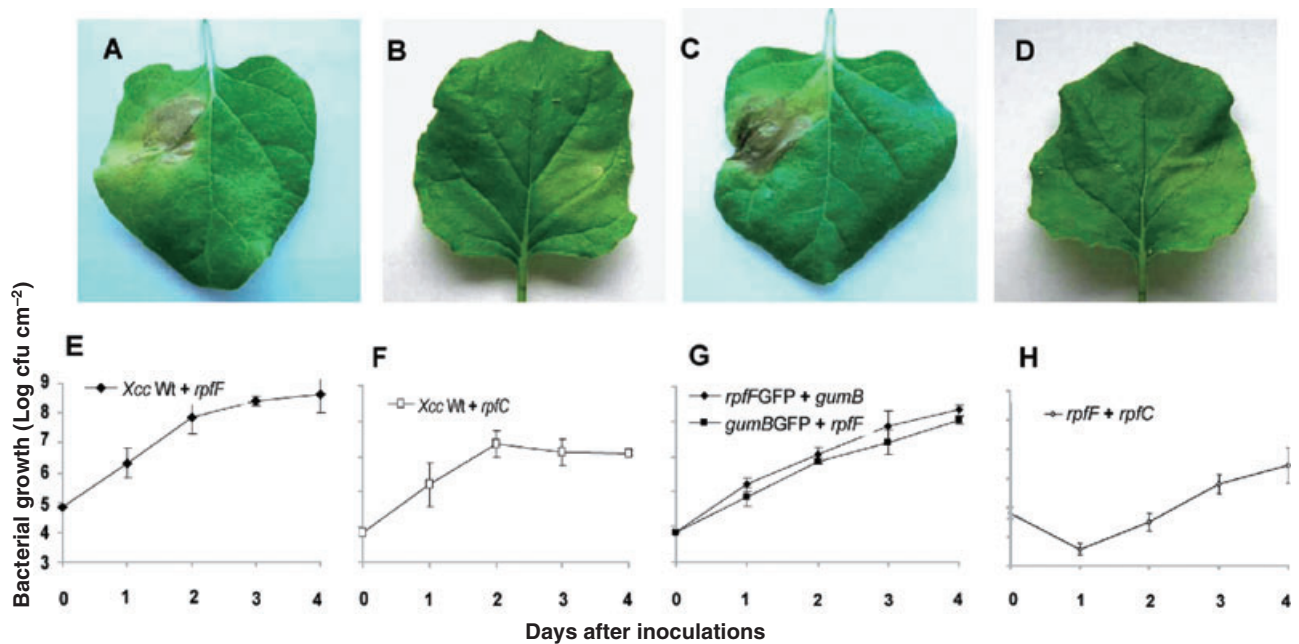
Fig. 5. Growth and symptom production by co-inoculated *Xcc* strains in *Nicotiana benthamiana*. Symptoms and growth were determined after inoculation into *N. benthamiana* leaves of a 1:1 mixture of *Xcc* strains. These mixed inocula were strains 8004 (wild type) and 8523 (*rpfF*) (A and E), strains 8004 (wild type) and 8557 (*rpfC*) (B and F), strains 8523 (*rpfF*) and 8397(*gumB*) (C and G) and strains 8523 (*rpfF*) and 8557(*rpfC*) (D and H). For all mixed inoculations, the numbers of the each bacterial strain were quantified after 4 days. In all cases the ratio remained close to 1:1.

ratio. The *rpfC* mutant interfered with symptoms caused by the wild-type 8004 and the total bacterial population was two orders of magnitude lower than that of the wild-type strain (Fig. 5B and F). In contrast, the presence of the DSF-defective *rpfF* mutant did not modify the symptoms and growth of wild-type strain 8004 in *N. benthamiana* leaves (Fig. 5A and E). Furthermore, co-inoculation of the *rpfC* and *rpfF* mutants allowed very limited bacterial growth and no symptoms were observed (Fig. 5D and H).

One possible interpretation of experiments with the *rpfC* mutant is that the elevated levels of DSF trigger plant defence responses that are responsible for the restriction of bacterial growth and symptom production. To address this point we examined the effects of inoculation of leaves of *N. benthamiana* with a DSF preparation on expression of a number of defence-related responses. DSF did not induce expression of the defence-related *PR1* gene or callose synthesis (data not shown), suggesting that the adverse effects of elevated levels of DSF on virulence are through direct effects on the co-inoculated wild-type bacteria.

Discussion

Cell–cell signalling and biofilm formation in different environments

The work in this article offers further insight into the role of the *rpf*/DSF signalling system in the biology of *Xcc*. By

examination of bacterial behaviour in static cultures in minimal medium we have demonstrated that DSF signalling has a role in the formation of structured biofilms and that an excess of DSF prevents such biofilm formation. This is a substantially different picture from that obtained from the study of aggregation/biofilm formation in shaken rich nutrient medium (Dow *et al.*, 2003). In this latter case, *rpf* mutants form matrix-enclosed aggregates whereas the wild-type strain does not. Furthermore, DSF causes aggregate dispersal in *rpfF* but not other *rpf* mutants. These experiments in rich nutrient medium suggested an effect of DSF on biofilm dispersal requiring the RpfC/RpfG two-component system, but no influence on biofilm formation.

Work on other bacteria has established that the environment has an impact on the contribution of cell–cell signalling or quorum sensing to the development of bacterial biofilms and that quorum sensing may be integral to biofilm formation only under certain conditions (Kjelleberg and Molin, 2002; Kirisits and Parsek, 2006). The same considerations appear to apply to the role of DSF signalling in biofilm formation in *Xcc*. In *Xcc* the perception of DSF is linked to the degradation of the intracellular signalling molecule cyclic di-GMP by the response regulator RpfG, an HD-GYP domain protein (Ryan *et al.*, 2006). Cellular levels of cyclic di-GMP are controlled through synthesis, catalysed by the GGDEF protein domain, and degradation by EAL and HD-GYP domains. The genome

of *Xcc* encodes 37 proteins with potential roles in cyclic di-GMP turnover. Many of these proteins contain additional signal transduction and sensory domains suggesting that their activities in cyclic di-GMP turnover are responsive to environmental cues (Ryan *et al.*, 2007). *Xcc* may thus integrate information from a number of environmental inputs, including cell–cell signalling, to modulate cellular cyclic di-GMP levels with consequent effects for biofilm formation and virulence factor synthesis (Ryan *et al.*, 2007). Consequently cell–cell signalling may not have a primary role in biofilm formation under all growth conditions. A further consideration is that the synthesis of xanthan, which is required for biofilm formation, is considerably enhanced in rich medium in the presence of glucose, so that mutation of *rpf* genes may reduce xanthan production below a critical level for biofilm formation only in minimal medium.

Cell–cell signalling and virulence in Xcc

The results of our experiments with mixed inoculations of bacteria indicate that DSF cell–cell signalling occurs *in planta*, but that an optimal concentration of DSF is required for virulence. A model of the production of DSF during the growth of *Xcc in planta* and its relationship to the production of virulence factors and the development of structured biofilms is shown in Fig. 6. Although a close correlation was observed between the effects of DSF levels on structured biofilm formation in minimal medium and on virulence, we cannot conclude a direct cause and effect relationship. Although there is no evidence to suggest that addition of excess DSF negatively influences the synthesis of extracellular enzymes or xanthan, we cannot exclude effects on the synthesis of other virulence determinants. Our findings are similar to those of Lindow and colleagues (2006) who have reported the effects of interference in *rpf* signalling in the virulence of *Xylella fastidiosa* the causal agent of Pierce's disease of grape. *Xylella fastidiosa* is closely related to *Xcc* and synthesizes a DSF-like signal molecule that is recognized by the *Xcc rpf* system but which is probably slightly different from DSF. Inoculation of bacteria able to degrade DSF or bacteria able to synthesize DSF (including an *rpfC* mutant of *X. fastidiosa*) can reduce virulence and symptom production by *X. fastidiosa* in grape. Lindow and colleagues concluded that DSF signalling was normally finely balanced during the disease process and that such a fine balance might therefore be readily disrupted. This may have substantial consequences for development of measures for the control of Pierce's disease. The role of *rpf*/DSF signalling in disease is somewhat different for *Xylella* and *Xanthomonas*. In particular, mutation of *rpfF* leads to enhancement of virulence in *X. fastidiosa* but reduced virulence in *Xcc*. Nevertheless, our findings suggest that
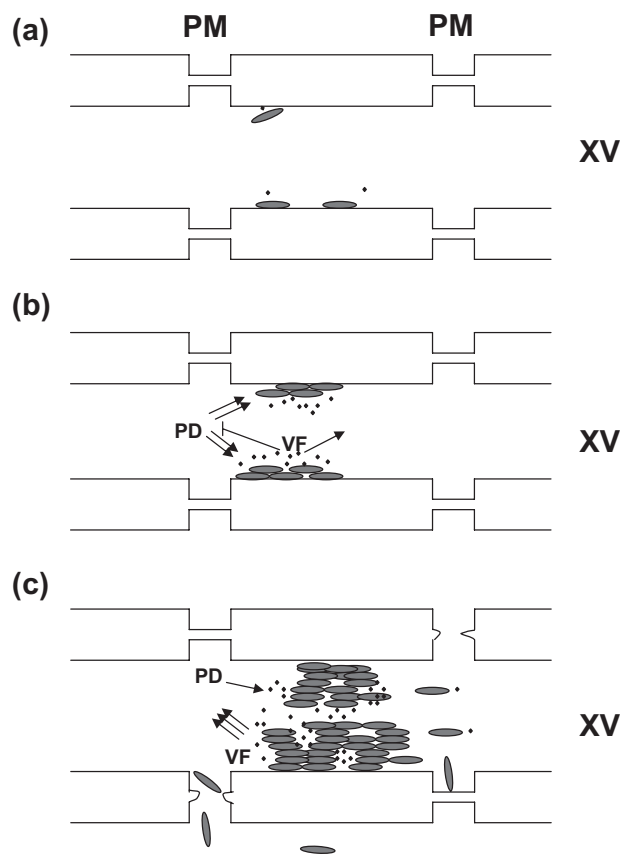

Fig. 6. Model of DSF production and its effect in biofilm formation and virulence factor production. At low bacteria cell density (A), where the production of DSF (diamonds) is limited, bacteria attach to the surfaces of the xylem vessels (XV). As the microcolony forms (B), DSF levels rise and the bacteria start to produce virulence factors (VF) including extracellular enzymes. Extracellular enzymes can promote disease through interference with plant defences (PD), provide nutrition through degradation of the xylem walls and allow passage of bacteria between xylem elements through degradation of bordered pit membranes (PM). In addition, the structured biofilm begins to form; bacteria within these structures may have increased resistance to host defences. At later stages (C), further elevation of DSF levels promotes biofilm dispersal, so that the bacteria can be released to colonize new tissue. The presence of elevated levels of DSF at early phases prevents the formation of the structured biofilm.

interference with DSF signalling may also have a role in the control of diseases caused by *Xcc* and perhaps other *Xanthomonas* spp.

Experimental procedures

Microbiological techniques

Xanthomonas campestris pv. *campestris* strains 8004 (wild type), 8523 (*rpfF*::Tn5lac) and 8557 (*rpfC*::pUIRM504) are previously described (Daniels *et al.*, 1984; Tang *et al.*, 1991; Slater *et al.*, 2000) and were grown at 28°C in PYM medium (Cadmus *et al.*, 1976) or in Y minimal medium containing glucose (1%, w/v) as the carbon source (Sherwood, 1970).

Escherichia coli was grown at 37°C in L medium (Sambrook *et al.*, 1989). Bacterial growth was monitored at 600 nm using MSE Spectroplus spectrophotometer. Plasmids were mobilized into *Xanthomonas* by triparental mating using a helper plasmid. For analysis of biofilm growth, bacteria were grown in PYM medium for 1 day [optical density at 600 nm (OD_{600}), about 1.5], and then the culture was used as an inoculum at a 1:1000 dilution in Y medium. Biofilm growth on glass was monitored in static cultures by confocal microscopy (see below). In some experiments, bacterial attachment to the side and at the bottom of the glass tubes and the wells of polystyrene plates was assayed by first growing the bacteria in shake flasks with Y medium to an OD_{600} of 0.8–1.0 and then pipetting 5 ml of this culture into 10 ml glass tubes or 2 ml into the wells of polystyrene 24-well flat-bottom tissue culture plates (Corning Incorporated, Corning, NY), which were then allowed to stand at 28°C for 48 h. Unbound bacteria were removed by gently washing the tubes or the wells three times with fresh growth medium, and attached bacteria were quantified by staining them with 0.01% (w/v) crystal violet (Acros Organics, Geel, Belgium), as described previously (O'Toole *et al.*, 1999).

Extraction and quantification of DSF from static cultures

DSF was extracted from culture supernatants using ethyl acetate as previously reported (Barber *et al.*, 1997). DSF was estimated by a bioassay in which restoration of endoglucanase production to an *rpfF* mutant is assessed (Barber *et al.*, 1997) Endoglucanase activity was measured by a radial diffusion assay and units were established by using a cellulase I enzyme (Sigma) as standard.

Nicotiana benthamiana *growth conditions and inoculations*

Nicotiana benthamiana seed germination and growth in soil were performed as previously reported (Yun *et al.*, 2006). All plant inoculations involved a minimum of three leaves from each of the three plants, and each experiment was carried out at least three times. Inoculation was performed according to published methods (Newman *et al.*, 1994). Bacteria were hand infiltrated into plant leaves at the abaxial surface by using a 1 ml syringe without needle, with *Xcc* strains (10^7 cfu ml^{-1} in H_2O) or H_2O, and bacterial development was assessed as reported (Yun *et al.*, 2006).

Confocal laser-scanning microscopy (CLSM)

A confocal laser-scanning microscope (Carl Zeiss LSM510-Axiovert 100 M) was used to visualize the different events of biofilm formation in a 4/5-day time-course experiment using chambered cover glass slides containing a borosilicate glass base 1 μm thick (Laboratory-Tek Nunc; No. 155411) and GFP-labelled bacteria as described previously (Russo *et al.*, 2006). Confocal images were acquired from bacterial cultures carrying the plasmid pRU1319, which expresses the green fluorescent protein (GFPuv) (Allaway *et al.*, 2001) or the plasmid pMP4518 expressing the enhanced yellow fluo-

rescent protein (EYFP) (Stuurman *et al.*, 2000). GFP- or EYFP-labelled bacterial cultures were diluted 1:1000 and grown in the chambers for up to at least 10 days at 28°C. Such static cultures typically reached an OD_{600} of about 1.7, as determined by re-suspending the biofilm bacteria and measuring their optical density. To prevent desiccation, the chambers were incubated in a humid sterile Petri dish. A typical mature biofilm was developed by the 8004 wild-type strain in static cultures in Y minimal medium containing glucose after 4/5 days at 28°C, when the OD_{600} was about 1.1. Three-dimensional images were reconstructed using the Zeiss LSM Image Browser version 3.2.0. Dual-colour confocal images were acquired from mixed cultures carrying the GFP-expressing plasmid (pRU1319) or the EYFP-expressing plasmid (pMP4518) (Stuurman *et al.*, 2000). GFP-expressing bacteria appeared green, and EYFP-expressing bacteria appeared in yellow. The detection of the emitted light was performed as described previously (Stuurman *et al.*, 2000). Dual-colour images were acquired by sequentially scanning with settings optimal for GFP (488 nm excitation with argon laser line and 505-nm-long pass emission) or EYFP (488 nm excitation with argon laser line and detection of emitted light between 530 and 600 nm). Rates of biofilm formation by bacteria expressing both constructs were similar, and no difference in growth or biofilm formation could be detected using (non-fluorescence) microscopy of biofilms formed by bacteria containing or lacking the GFP or EYFP construct.

References

Allaway, D., Schofield, N.A., Leonard, M.E., Gilardoni, L., Finan, T.M., and Poole, P.S. (2001) Use of differential fluorescence induction and optical trapping to isolate environmentally induced genes. *Environ Microbiol* **3:** 397–406.

Barber, C.E., Tang, J.L., Feng, J.X., Pan, M.Q., Wilson, T.J., Slater, H., *et al.* (1997) A novel regulatory system required for pathogenicity of *Xanthomonas campestris* is mediated by a small diffusible signal molecule. *Mol Microbiol* **24:** 555–566.

Cadmus, M.C., Rogovin, S.P., Burton, K.A., Pittsley, J.E., Knutson, C.A., and Jeanes, A. (1976) Colonial variation in *Xanthomonas campestris* NRRL B-1459 and characterization of the polysaccharide from a variant strain. *Can J Microbiol* **22:** 942–948.

Crossman, L., and Dow, J.M. (2004) Biofilm formation and dispersal in *Xanthomonas campestris*. *Microbes Infect* **6:** 623–629.

Daniels, M.J., Barber, C.E., Turner, P.C., Sawczyc, M.K., Byrde, R.J., and Fielding, A.H. (1984) Cloning of genes involved in pathogenicity of *Xanthomonas campestris* pv. *campestris* using the broad host range cosmid pLAFR1. *EMBO J* **3:** 3323–3328.

Dow, J.M., and Daniels, M.J. (1994) Pathogenicity determinants and global regulation of pathogenicity of *Xanthomonas campestris* pv. *campestris*. *Curr Top Microbiol Immunol* **192:** 29–41.

Dow, J.M., Crossman, L., Findlay, K., He, Y.Q., Feng, J.X., and Tang, J.L. (2003) Biofilm dispersal in *Xanthomonas campestris* is controlled by cell–cell signaling and is required for full virulence to plants. *Proc Natl Acad Sci USA* **100:** 10995–11000.

Kirisits, M.J., and Parsek, M.R. (2006) Does *Pseudomonas aeruginosa* use intercellular signalling to build biofilm communities? *Cell Microbiol* **8:** 1841–1849.

Kjelleberg, S., and Molin, S. (2002) Is there a role for quorum sensing signals in bacterial biofilms? *Curr Opin Microbiol* **5:** 254–258.

Lindow, S.E., Chatterjee, S., and Purcell, A. (2006) Management of Pierce's disease of grape by interfering with cell-cell communication in *Xylella fastidiosa* [WWW document]. URL http://www.cdfa.ca.gov/phpps/pdcp/ResearchSymposium/2006Section3.pdf.

Newman, M.A., Conrads-Strauch, J., Scofield, G., Daniels, M.J., and Dow, J.M. (1994) Defense-related gene induction in *Brassica campestris* in response to defined mutants of *Xanthomonas campestris* with altered pathogenicity. *Mol Plant Microbe Interact* **7:** 553–563.

Onsando, J. (1992) Black rot of crucifers. In *Diseases of Vegetables and Oil Seed Crops.* Chaube, H.S., Kumar, J., Mukhopadhyay, A.N., Singh, U.S. (eds). New Jersey: Prentice Hall, pp. 243–252.

O'Toole, G.A., Pratt, L.A., Watnick, P.I., Newman, D.K., Weaver, V.B., and Kolter, R. (1999) Genetic approaches to study of biofilms. *Methods Enzymol* **310:** 91–109.

Russo, D.M., Williams, A., Edwards, A., Posadas, D.M., Finnie, C., Dankert, M., *et al.* (2006) Proteins exported via the PrsD-PrsE type I secretion system and the acidic exopolysaccharide are involved in biofilm formation by *Rhizobium leguminosarum*. *J Bacteriol* **188:** 4474–4486.

Ryan, R.P., Fouhy, Y., Lucey, J.F., Crossman, L.C., Spiro, S., He, Y.W., *et al.* (2006) Cell–cell signaling in *Xanthomonas campestris* involves an HD-GYP domain protein that functions in cyclic di-GMP turnover. *Proc Natl Acad Sci USA* **103:** 6712–6717.

Ryan, R.P., Fouhy, Y., Lucey, J.F., Jiang, B.-L., He, Y.-Q., Feng, J.X., *et al.* (2007) Cyclic di-GMP signalling in the virulence and environmental adaptation of *Xanthomonas campestris*. *Mol Microbiology* **63:** 429–442.

Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY, USA: Cold Spring Harbor Laboratory Press.

Sherwood, M. (1970) Improved synthetic medium for the growth of Rhizobium. *J Appl Bacteriol* **33:** 708–713.

Slater, H., Alvarez-Morales, A., Barber, C.E., Daniels, M.J., and Dow, J.M. (2000) A two-component system involving an HD-GYP domain protein links cell–cell signalling to pathogenicity gene expression in *Xanthomonas campestris*. *Mol Microbiol* **38:** 986–1003.

Stuurman, N., Pacios Bras, C., Schlaman, H.R., Wijfjes, A.H., Bloemberg, G., and Spaink, H.P. (2000) Use of green fluorescent protein color variants expressed on stable broad-host-range vectors to visualize rhizobia interacting with plants. *Mol Plant Microbe Interact* **13:** 1163–1169.

Tang, J.L., Liu, Y.N., Barber, C.E., Dow, J.M., Wootton, J.C., and Daniels, M.J. (1991) Genetic and molecular analysis of a cluster of rpf genes involved in positive regulation of synthesis of extracellular enzymes and polysaccharide in *Xanthomonas campestris* pathovar campestris. *Mol Gen Genet* **226:** 409–417.

Vojnov, A.A., Zorreguieta, A., Dow, J.M., Daniels, M.J., and Dankert, M.A. (1998) Evidence for a role for the *gumB* and *gumC* gene products in the formation of xanthan from its pentasaccharide repeating unit by *Xanthomonas campestris*. *Microbiology* **144** (Part 6)**:** 1487–1493.

Vojnov, A.A., Slater, H., Daniels, M.J., and Dow, J.M. (2001) Expression of the gum operon directing xanthan biosynthesis in *Xanthomonas campestris* and its regulation in planta. *Mol Plant Microbe Interact* **14:** 768–774.

Vojnov, A.A., Bassi, D.E., Daniels, M.J., and Dankert, M.A. (2002) Biosynthesis of a substituted cellulose from a mutant strain of *Xanthomonas campestris*. *Carbohydr Res* **337:** 315–326.

Wang, L.H., He, Y., Gao, Y., Wu, J.E., Dong, Y.H., He, C., *et al.* (2004) A bacterial cell–cell communication signal with cross-kingdom structural analogues. *Mol Microbiol* **51:** 903–912.

Yun, M.H., Torres, P.S., El Oirdi, M., Rigano, L.A., Gonzalez-Lamothe, R., Marano, M.R., *et al.* (2006) Xanthan induces plant susceptibility by suppressing callose deposition. *Plant Physiol* **141:** 178–187.

Supplementary material

The following supplementary material is available for this article online:
Fig. S1. Relative levels of DSF in strains of *Xcc* grown in static culture in Y medium supplemented with glucose as a function of time of growth. DSF was assayed by restoration of endoglucanase activity to an *Xcc rpfF* mutant (for details see *Experimental procedures*). The *rpfF* mutant still has some residual endoglucanase activity, but no DSF activity. The *rpfC* mutant has significantly higher levels of DSF than the wild type, from day 2 onwards.
Fig. S2. Relative levels of xanthan in strains of *Xcc* grown in static culture in Y medium supplemented with glucose as a function of time of growth. Xanthan levels, which are expressed as mg ml^{-1}, are significantly higher in the wild type than in *rpfF* and *rpfC* mutants after 2 days of growth, consistent with previous data showing positive regulation by the DSF/*rpf* system in *Xcc*.

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