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XbmR, a new transcription factor involved in the regulation of chemotaxis, biofilm formation and virulence in *Xanthomonas citri* subsp. *citri*

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Summary

***Xanthomonas citri* subsp. *citri* (*Xcc*) is the causal agent of citrus canker. Biofilm formation on citrus leaves plays an important role in epiphytic survival of *Xcc*. Biofilm formation is affected by transposon insertion in XAC3733, which encodes a transcriptional activator of the NtrC family, not linked to a gene encoding a sensor protein, thus could be considered as an 'orphan' regulator whose function is poorly understood in *Xanthomonas* spp. Here we show that mutation of XAC3733 (named *xbmR*) resulted in impaired structural development of the *Xcc* biofilm, loss of chemotaxis and reduced virulence in grapefruit plants. All defective phenotypes were restored to wild-type levels by the introduction of PA2567 from *Pseudomonas aeruginosa*, which encodes a phosphodiesterase active in the degradation of cyclic diguanosine monophosphate (c-di-GMP). A knockout of *xbmR* led to a substantial downregulation of *fliA* that encodes a σ^{28} transcription factor, as well as *fliC* and XAC0350 which are potential member of the σ^{28} regulon. XAC0350 encodes an HD-GYP domain c-di-GMP phosphodiesterase. These findings suggest that XbmR is a key regulator of flagellar-dependent motility and chemotaxis exerting its action through a regulatory pathway that involves FliA and c-di-GMP.**

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Introduction

Citrus canker is one of the most important and aggressive bacterial diseases of citrus trees. It is present in more than 30 citrus-producing countries and causes significant losses worldwide. The causal agent of this disease is *Xanthomonas citri* subsp. *citri* (*Xcc*). This bacterium enters the leaves of the host plant through stomata or tissue lesions; typical canker lesions can be found on leaves, stems and fruits and are distinguished by a necrotic area surrounded by oily, water-soaked margins and yellow chlorotic rings (Brunings and Gabriel, 2003).

Epiphytic bacteria, including pathogens such as *Xcc* that have an epiphytic phase to their disease cycle, must be able to tolerate considerable environmental stresses (Yu *et al*., 2013). Leaf surfaces are continually exposed to extreme conditions such as lack of moisture, ultraviolet irradiation, strong winds and heat. Bacterial tolerance of these stressful conditions is often associated with the formation of biofilms. The first step in biofilm formation is bacterial initial attachment, a process that can be mediated by flagella. Following this first step, microorganisms form microcolonies that can further develop into mature structured biofilms. The biofilm 'cycle' is completed when mature biofilms disperse to release motile cells (O'Toole and Kolter, 1998; Lemon *et al*., 2007).

A number of factors have already been implicated in the formation of *Xcc* biofilms on abiotic or biotic surfaces. These include the extracellular polysaccharide xanthan, lipopolysaccharides, the periplasmic glucan synthase HrpM, both flagellum-dependent and flagellum-independent cell motility, secreted proteins and extracellular DNA (Siciliano *et al*., 2006; Rigano *et al*., 2007; Li and Wang, 2011; Malamud *et al*., 2012). Multiple

signalling and regulatory pathways are also involved, to include cell–cell signalling mediated by the diffusible signal factor (DSF) and pathways involving the second messenger cyclic diguanosine monophosphate (c-di-GMP) (O'Connell *et al*., 2013). Despite these insights, our understanding of biofilm formation in *Xcc* and its regulation is far from complete.

Previous work from our laboratory described a genetic screen for biofilm formation-defective mutants in *Xcc* (Malamud *et al*., 2013). This work identified that a mutant with a transposon insertion in XAC3733 (named XbmR, *Xanthomonas* biofilm and motility regulator) had significantly reduced attachment to a polystyrene surface but an indistinguishable growth rate from that of the wild type in Y minimal medium (YMM) medium (Malamud *et al*., 2013). Preliminary bioinformatic analysis indicated that XAC3733 belongs to the NtrC family of transcriptional activators that are known to control a variety of physiological processes in diverse bacteria in response to environmental signals (Andrade *et al*., 2006; De Carlo *et al*., 2006; Kim and McCarter, 2007; Kroos, 2007). In general, members of the NtrC family have three primary domains (De Carlo *et al*., 2006): a conserved central domain that hydrolyses ATP and activates transcription by interacting with σ^{54}-RNA polymerase, an N-terminal two-component response regulator receiver domain and a C-terminal domain containing a DNA-binding motif. This family of activators has been shown to regulate σ^{54}-dependent promoters in some bacterial species (Hirschman *et al*., 1985; Kustu *et al*., 1989; Studholme and Dixon, 2003) and has been recognized as enhancer-binding proteins in phosphorylated forms (De Carlo *et al*., 2006).

There are few reports of the role of NtrC family regulators in *Xanthomonas* spp. (Wang *et al*., 2011; Malamud *et al*., 2013) and the function of the XAC3733 in *Xcc* has not been investigated in depth. Here, we present evidence that the XAC3733 is involved in biofilm formation and pathogenesis of *Xcc* through regulation of the synthesis of flagella and chemotaxis. Moreover, we provide data suggesting a possible role of c-di-GMP in XmbR regulation of these functions.

Results

XAC3733 is an NtrC family protein

The predicted amino acid sequence of XAC3733 was compared with sequences of the two other NtrC family proteins in *Xcc* (XAC0208 and XAC0226) as well as NtrC family proteins in *Xanthomonas oryzae* pv. *oryzae* (XOO4483), *Xanthomonas campestris* pv. *campestris* (XCC0563), *Pseudomonas putida* (PP_5048), *Pseudomonas aeruginosa* (PA5125), *Vibrio vulnificus* (VV1_0892), *Agrobacterium tumefaciens* (AGRO_4553) and *Stenotrophomonas maltophilia* (Smal_0123) (Fig. 1). The amino acid sequence of XAC3733 shares 40% identity with XAC0208, 35% with XAC0226, 39% with XOO4483 and Smal_0123, 38% with XCC0563 and 34% with PP_5048, PA5125, VV1_0892 and AGRO_4553. Notably, the gene products in *V. vulnificus* (VV1_0892) and in *A. tumefaciens* (AGRO_4553) have already been implicated in biofilm formation (Kim *et al*., 2009a,b; Ruffing and Chen, 2012).

The AAA+ domain of NtrC family proteins has ATPase activity associated with a wide variety of cellular activities (De Carlo *et al*., 2006). There is a GAFTGA motif in this domain that is implicated in the binding with the σ^{54} subunit of the RNA polymerase and consequent activation in RNA synthesis (De Carlo *et al*., 2006). There are differences in the GAFTGA motif between *Xanthomonas* spp. In particular, XAC3733 has the variant GSFTGA with substitution of an alanine residue by serine (Fig. 1).

Analysis of the genome sequence suggests that the *xbmR* gene is monocistronic and transposon insertion does not therefore have a polar effect (Fig. S1). Unlike XAC0208 and XAC0226, which encode the two other NtrC family proteins in *Xcc*, XAC3733 is not linked to a gene encoding a sensor protein, and can thus be considered as an 'orphan' regulator. Interestingly, we identified in this protein a putative phosphorylation site in Asp-64 residue, which is similar to the phosphorylation site (Asp-55) found in NtrC of *P. putida* by Hervas and colleagues (2009).

The xbmR *mutant has an altered biofilm structure*

The role of the XbmR protein in the structural development of *Xcc* biofilms was studied in detail by observing the *Xcc* biofilm development with confocal laser scanning microscope (CLSM). Wild type, mutant and complemented mutant strains were stained with the BacLight LIVE/DEAD viability kit. The CLSM images revealed the presence of a mature biofilm after a 4 day time-course experiment of static cultures of the wild-type strain (Fig. 2A). In contrast, the *xbmR* mutant showed the absence of a typical biofilm of the wild type with reduced biofilm biomass and thickness as revealed by COMSTAT analysis (Table 1). Complementation with XAC3733 cloned in pBBR1-MCS to give the *c-xbmR* strain (see *Experimental procedures*) led to an increase in biofilm biomass and thickness, although full restoration to the level of the wild-type strain was not observed (Table 1; Fig. 2A).

The effect of xbmR *mutation on motility and chemotaxis*

Flagellar dependent motility is one of the determinants for biofilm formation in many bacteria (Malamud *et al*.,

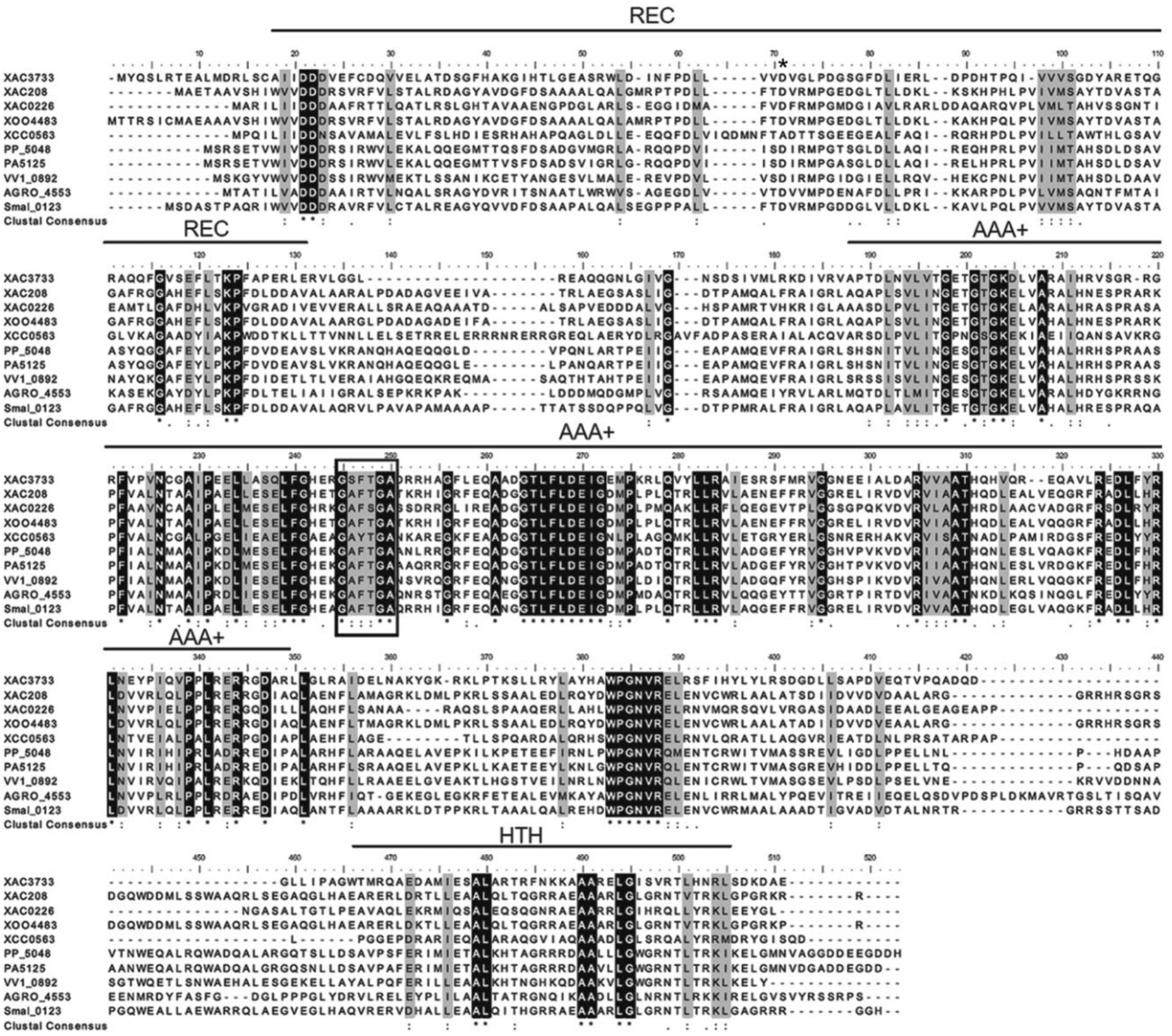

Fig. 1. Multiple alignment of the NtrC family proteins in *Xcc* with their homologues in others organisms. Sequence alignment was performed by using CLUSTALW. Identical and conserved residues are shown in dark background. Asterisks indicate identical residues; highly conserved (:) and less conserved (.). The GenBank accession numbers of the NtrC proteins are: *Xcc* (XAC3733) NP_644040.1; (XAC0208) NP_640564.1; (XAC0206) NP_640582.1, for *X. oryzae* pv. *oryzae* (XOO4483) YP_203122.1; *X. campestris* pv. *campestris* (XCC0563) NP_635955.1; *P. putida* (PP_5048) AAN70613.1; *P. aeruginosa* (PA5125); NP_253812.1; *V. vulnificus* (VV1_0892) NP_759868.1; *A. tumefaciens* (AGRO_4553) EGL62776.1 and *S. maltophilia* (Smal_0123) YP_002026511.1. The region inside the box is a known conserved motif of the AAA+ domain. Asterisk indicate the putative phosphorylation site.

2011; Guttenplan and Kearns, 2013). In order to examine any relationship between biofilm development, motility and chemotaxis in *Xcc*, we performed an assay to measure bacteria movement towards grapefruit leaf extract. Grapefruit extracts elicited a detectable chemotactic response in *Xcc* wild-type strain that was not detectable in the *xbmR* mutant. The chemotactic response of this mutant strain was restored by complementation with an intact copy of the *xbmR* gene and its promoter region (*c-xbmR*; Fig. 2B).

XbmR is implicated in the regulation of flagellar synthesis

The absence of a chemotactic response in the *xbmR* mutant could result from impairment in the ability to sense chemotactic signals or from impairment in flagellar action or biogenesis leading to a loss of motility *per se*. Examination of the flagellar structure of the strains using transmission electron microscopy showed a single polar flagellum in the wild-type strain, but no flagellum in the

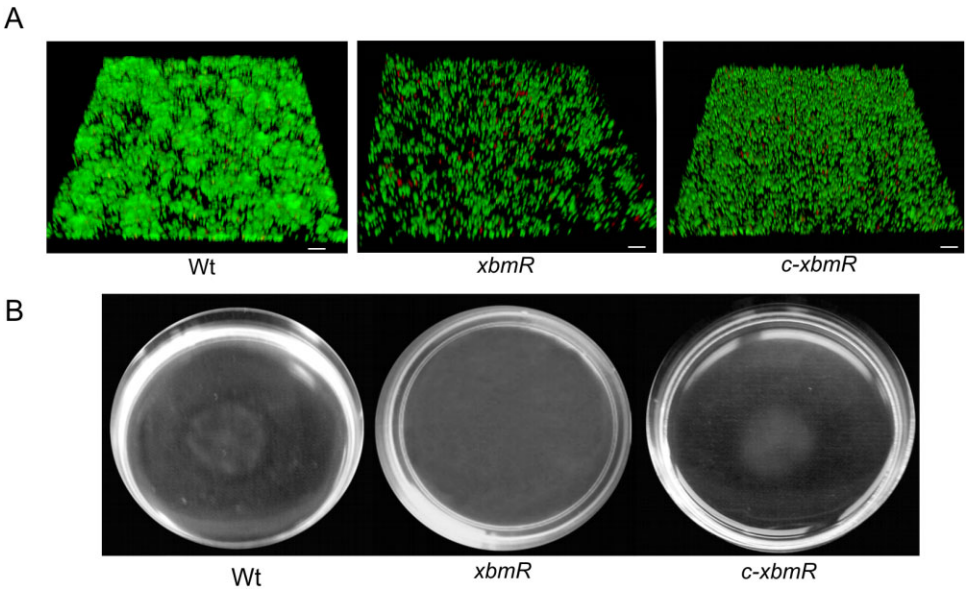

Fig. 2. Biofilm formation and chemotaxis of *Xcc* strains. Biofilm structures were analysed using confocal laser scanning microscopy (CLSM) for *Xcc* wild type 306 (WT), *xbmR* mutant and the complemented *c-xbmR* strains. Cells were grown in chambered cover slides for 4 days, then stained using the BacLight LIVE/DEAD viability kit and observed by CLSM. Scale bars 10 μm (A). Effect of the mutation of *xbmR* gene and the complemented strain *c-xbmR* on the chemotactic response to grapefruits extract in *Xcc*. Ten microlitre of extract was placed in the centre of each plate. Photographs were taken after incubation for 30 min to 1 h (B).

xbmR mutant. The *c-xbmR* recovered the flagellar structure (Fig. 3A). Chemotaxis assays together with swimming motility assays (Fig. S3) reinforce that *xbmR* is unable to move due to the absence of flagella. These observations suggested a role for NtrC1 in regulation of flagella biosynthesis, which was subsequently investigated in greater detail.

The expression of flagellar genes is controlled in a hierarchical manner by several regulators. These regulators can be classified into three classes. Class I include master regulators such as *rpoN2*, *fleQ* and *flgM*, which regulate class II genes, such as FliA. This transcription factor controls the expression of several class III genes, which include *fliC* that encodes flagellin (Yang *et al*., 2009).

The comparative level of expression of some of the most important genes related to the flagellum synthesis between the wild-type strain and the *xbmR* mutant was determined using quantitative polymerase chain reaction (qRT-PCR). The results showed about a 20-fold decrease in expression of *fliC* in the *xbmR* mutant when compared with the wild-type strain (Fig. 3B). The levels of expression of *fliA* and *flgM* were also significantly lower in the *xbmR* mutant compared with the wild type (by about 18-fold and 7-fold respectively) (Fig. 3B). In contrast, no substantial differences were observed in the expression level of *rpoN2* and *fleQ* between the wild type and the *xbmR* mutant strain (Fig. 3B).

XbmR regulation is linked to c-di-GMP metabolism

The second messenger c-di-GMP has been implicated in regulation of biofilm formation, motility and chemotaxis in a number of bacteria (Romling, 2012; Sondermann *et al*., 2012). The phenotypic effects of *xbmR* mutation on biofilm formation and motility in *Xcc* prompted us to

Table 1. Evaluation of biofilm properties by COMSTAT.

Strain	Biomass ($\mu m^3\ \mu m^{-2}$)	Average thickness (μm)	Roughness coefficient
Wt	5.70 ± 0.021 (a)	7.15 ± 0.1 (a)	0.30 ± 0.04 (a)
xbmR	1.72 ± 0.10 (b)	2.90 ± 0.06 (b)	1.13 ± 0.01 (b)
c-xbmR	3.92 ± 0.15 (c)	6.03 ± 0.04 (a)	0.57 ± 0.09 (a)
***xbmR* (PL2567)**	4.05 ± 0.40 (c)	6.25 ± 0.30 (a)	0.51 ± 0.12 (a)
***xbmR* (*wspR*19)**	2.10 ± 0.10 (b)	3.7 ± 0.34 (b)	1.06 ± 0.01 (b)

All data represent the mean ± standard deviation; different letters indicate significant differences ($P < 0.05$).
Measurements of total biomass, average thickness and roughness coefficient of 4-day-old biofilm.

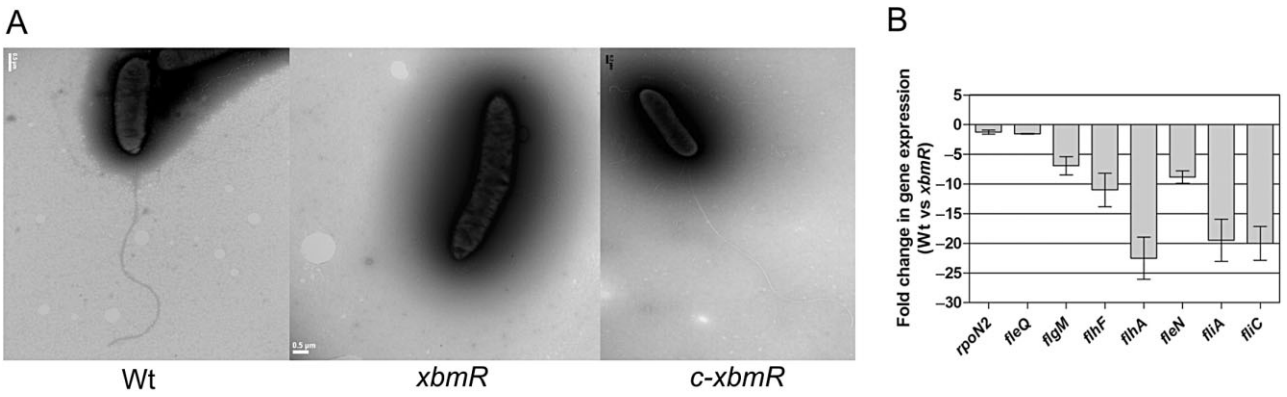

Fig. 3. Analysis of the effects of *xbmR* mutation on flagella structure and flagellar gene expression. Transmission electron microscopy was performed to examine the presence of flagella in *Xcc* wild type 306, *xbmR* and *c-xbmR* strains grown in PYM broth without glucose. Scale bars, 0.5 μm (A). Relative expression levels of the flagella genes (including *fliC*, *fliA*, *flgM*, *flhF*, *flhA*, *fleN*, *fleQ* and *rpoN2*) in the *Xcc* wild type 306 and the *xbmR* mutant were determined by qRT-PCR (B). Data presented are averages of three independent experiments. Error bars represent the standard deviation of the samples.

examine a possible regulatory relationship between XbmR and c-di-GMP metabolism.

In the first round of experiments, the effects of artificially changing the c-di-GMP level on different phenotypes of the wild type and *xbmR* mutant of *Xcc* were examined. This alteration in c-di-GMP was achieved by exogenous expression of either the diguanylate cyclase WspR19 from *Pseudomonas fluorescens* or the c-di-GMP phosphodiesterase PA2567 from *P. aeruginosa* (Ryan *et al*., 2006).

Biofilm produced by the different strains were examined by CLSM and the images were also analysed by COMSTAT to determine the total biomass, average thickness and roughness coefficient. Expression of PA2567 partially restored the wild-type biofilm formation and characteristics to the *xbmR* mutant (Fig. 4; Table 1). In contrast, the expression of WspR19 in the *xbmR* mutant did not produce apparent significant changes in biofilm formation (Fig. 4). Accordingly, the values for biomass and thickness for the biofilm of *xbmR* and *xbmR* (*wspR19*) were significantly lower than those for the wild-type strain and *xbmR* (*PA2567*). The roughness coefficient indicates the heterogeneity of the biofilm, considering how its thickness varies (Murga *et al*., 1995); high values indicate more biofilm heterogeneity. Biofilms produced by the *xbmR* and *xbmR* (*wspR19*) strains showed higher values than the wild type and *xbmR* (*PA2567*) strains (Table 1). This result was consistent with the contention that *xbmR* and *xbmR* (*wspR19*) were not able to form the mature biofilm of the wild type (Fig. 4). Expression of WspR19 in wild-type *Xcc* strain produced a loss of biofilm formation whereas expression of PA2567 did not produce any effect (data not shown).

The motility phenotype and related gene expression in the different strains was also examined. Expression of PA2567 in the *Xcc xbmR* mutant restored *fliC* expression to levels close to those of wild-type strain (Fig. 5C) and restored chemotaxis (Fig. 5A). In contrast, expression of WspR19 protein had no effect on *fliC* expression or chemotaxis in the *xbmR* mutant (Fig. 5A and C). The presence of flagella in all the strains was assessed by optical microscopy. Flagella were observed for the wild type and for the *xbmR* (PA2567), while no flagellum was observed for *xbmR* and *xbmR* (*wspR*19) strains (Fig. 5B).

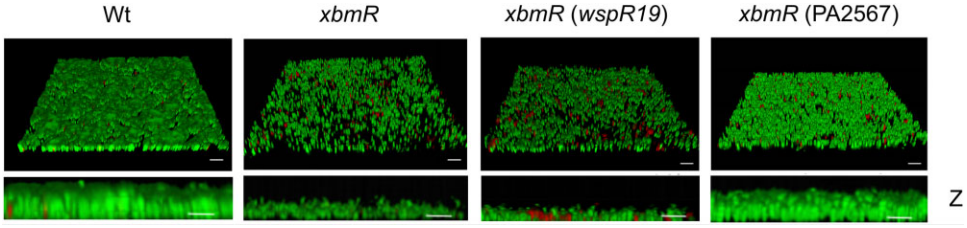

Fig. 4. Effects of modulation of c-di-GMP level on biofilm formation by the *xbmR* mutant. C-di-GMP levels in the *xbmR* mutant were altered by expression of PA2567 (with c-di-GMP phosphodiesterase activity) or WspR19 (with diguanylate cyclase activity). Biofilms formed by these strains designated *xbmR* (WSPR19) and *xbmR* (PL2567) are shown alongside the *Xcc* wild type 306 (WT) and the *xbmR* mutant (*xbmR*). z-axis projected images. Scale bars = 5 μm.

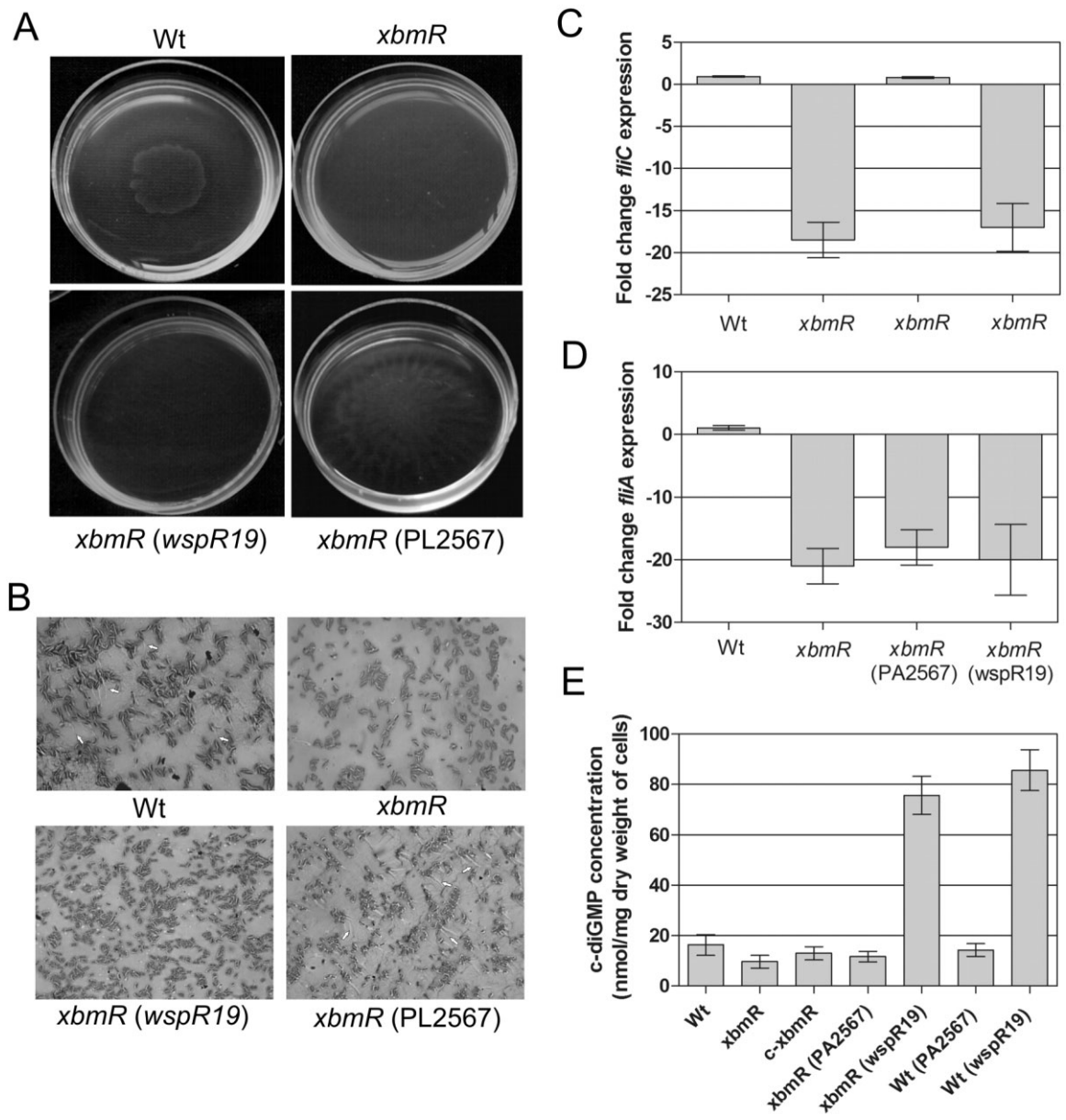

Fig. 5. Effect of modulation of c-di-GMP level on chemotaxis and flagella synthesis and gene expression in Xcc. C-di-GMP levels in the *xbmR* mutant were altered by expression of PA2567 (with c-di-GMP phosphodiesterase activity) or WspR19 (with diguanylate cyclase activity). Chemotactic response of the different *Xcc* strains in comparison to wild type 306 (WT) and the *xbmR* mutant shown (A). Flagellum staining and visualization by optical microscope at 1000× magnification. Arrows indicate bacterial flagellum (B). *fliC* expression in the *Xcc* wild type 306 (WT), *xbmR* mutant, *xbmR* (PL2567) and *xbmR* (wspR19) (C). *fliA* expression in the *Xcc* wild type 306 (WT), *xbmR* mutant, *xbmR* (PL2567) and *xbmR* (wspR19) (D). Data presented are averages of three independent experiments. Error bars represent the standard deviation of the samples. C-di-GMP measurement in the *Xcc* wild type 306 (WT), *xbmR* mutant, *xbmR* (PL2567) and *xbmR* (wspR19), *Xcc* wild type 306 (PL2567), *Xcc* wild type 306 (wspR19) and *c-xbmR* (E). Data presented are the means and standard deviations of three independent experiments.

Expression of WspR19 in wild type *Xcc* strain produced loss of chemotaxis whereas expression of PA2567 did not produce any effect (Fig. S2).

The effects of expression of WspR19 and PA2567 on the biofilm and motility phenotypes prompted us to measure the cellular levels of c-di-GMP in the wild type and *xbmR* mutant strains. Under the conditions tested, the level of c-di-GMP in the *xbmR* mutant was not statistically different from that of the wild type (Fig. 5E). Furthermore, expression of PA2567 had no significant effect on the level of the c-di-GMP in either strain. In contrast, expression of WspR19 gave a large and highly significant

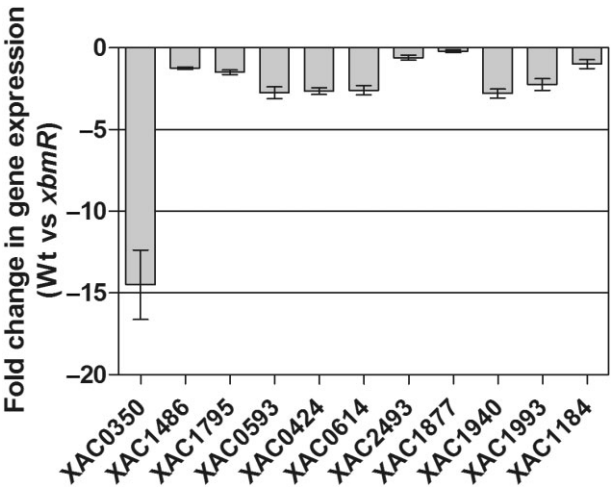

Fig. 6. Effects of mutation of *xbmR* on expression of genes encoding EAL, HD-GYP or GGDEF domains related to c-di-GMP metabolism. XAC0350, XAC1877 (RpfG) and XAC2493 are HD-GYP domain proteins; XAC0424, XAC0593, XAC0614, XAC1486 and XAC1940 are GGDEF domain proteins; XAC1184 and XAC1993 are EAL domain proteins, whereas XAC1795 has an EAL domain and a degenerate GGDEF domain. Data presented are the fold change expression in the *xbmR* mutant compared with wild type and are averages of three independent experiments. Error bars represent the standard deviation of the samples.

increase in c-di-GMP level in both the wild type and *xbmR* mutant background (Fig. 5E). Thus although mutation of *xbmR* has an effect on biofilm formation and motility, this is not manifested in a significant overall increase in the level of cyclic di-GMP. Nevertheless, the phenotypic reversal following expression of PA2567 suggests that *xbmR* mutation does affect the nucleotide levels, perhaps in a localized fashion. Along the same lines, expression of WspR19 in the wild type gives a high cellular level of the nucleotide, which perhaps swamps all the responsive systems, thus giving a phenocopy of the *xbmR* mutant (Fig. S2).

The expression of an HD-GYP domain-containing protein is dependent on XbmR

The influence of XbmR on the level of c-di-GMP in *Xcc* could be exerted in a number of different ways. One possible mechanism is through the action of FliA. In the *xbmR* mutant strain, *fliA* is downregulated (Fig. 3B). Furthermore, a bioinformatic analysis of the potential FliA regulon in *X. campestris* identified that a number of possible target genes encode products implicated in synthesis or degradation of c-di-GMP (Yang *et al*., 2009). Specifically, these products were identified as five GGDEF domain-containing proteins and one HD-GYP domain-containing c-di-GMP phosphodiesterase (Yang *et al*., 2009). We identified the homologues of these five GGDEF and one HD-GYP domain-containing proteins in *Xcc*. Of these homologues, XAC0424, XAC0593, XAC0614 and XAC1486 contain canonical sequences (including the GGDEF motif) associated with active diguanylate cyclases. In contrast, XAC1795 contains a degenerate GGDEF domain (with the sequence DPTTF replacing GGDEF), but has an EAL domain which is likely to be active in c-di-GMP degradation. The HD-GYP domain protein XAC0350 contains conserved amino acid residues required for metal binding and the binding and degradation of cyclic di-GMP (Bellini *et al*., 2014) suggesting that it is enzymatically active.

Analysis of the promoters of the encoding genes in *Xcc* revealed the presence of motifs associated with class III promoters (Table S2) as was found in *X. campestris*. Analysis of the full genome sequence of *Xcc* revealed further potential members of the FliA regulon (Table S2), to include several genes that encode additional c-di-GMP signalling proteins: XAC1184 (EAL domain) and XAC1940 (GGDEF domain).

The relative level of expression of these *Xcc* genes in the wild type and *xbmR* mutant was evaluated by qRT-PCR. The data demonstrated that the expression of XAC0350 was reduced by about 14-fold in the *xbmR* mutant in comparison with the wild-type strain, whereas the genes encoding the GGDEF domain proteins were reduced by ≤ 2.5-fold (Fig. 6). Expression of XAC1184 encoding an EAL domain protein was not altered between wild type and *ntrC1* mutant. In addition, we analysed the expression of genes encoding other proteins implicated in c-di-GMP turnover [the HD-GYP domain proteins XAC1877 (RpfG) and XAC2493] that were predicted not to be targets of FliA regulation. The expression of these genes was unaltered in the *xbmR* mutant.

The activity of XAC0350 as a c-di-GMP phosphodiesterase was examined by expression in the *rpfG* mutant of *X. campestris*. In this strain, the high cellular level of c-di-GMP leads to a repression of synthesis of endoglucanase; expression of active phosphodiesterases gives a de-repression of enzyme synthesis (Ryan *et al*., 2006; 2009). Expression of the HD-GYP domain of XAC0350 (see *Experimental procedures*) led to a small but significant increase in the production of endoglucanase in the *rpfG* mutant background (Table S3), indicating that XAC0350 is active as a c-di-GMP phosphodiesterase.

The differential effect on expression of genes encoding diguanylate cyclases and phosphodiesterases may contribute to the elevated level of c-di-GMP that is presumed to occur locally in the *xbmR* mutant. The expression of PA2567 and WspR19 in the *xbmR* mutant did not restore the *fliA* expression levels to those of the wild-type strain (Fig. 5D), suggesting that the regulatory action of c-di-GMP is exerted downstream of *fliA* expression.

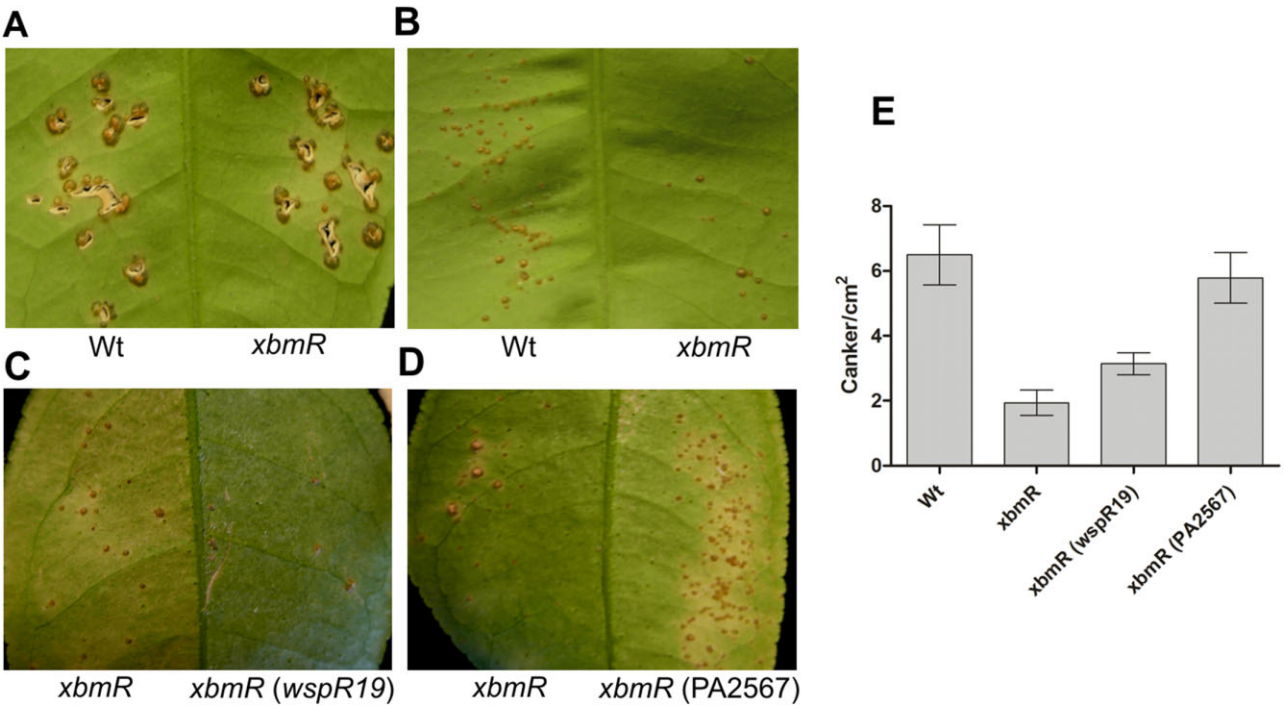

Fig. 7. Pathogenicity assays. 1×10^6 cfu ml^{-1} bacterial suspension of the *Xcc* wild type 306 (WT) and the *xbmR* mutant were swabbed on grapefruits leaves previously injured (A). 1×10^6 cfu ml^{-1} bacterial suspension of the *Xcc* wild type 306 (WT), *xbmR* mutant, *xbmR* (wspR19) and *xbmR* (PA2567) were swabbed on grapefruits healthy leaves (B), (C) and (D). Pictures were taken 4 weeks after infection. Three independent experiments were carried out for each strain with similar results. Measurement of the number of cankers per cm^2 of grapefruit leaves at 20 dpi. Twenty leaves were assayed and the results are the means of four independent experiments. Error bars represent the standard deviation of the samples (E).

Virulence is affected in the xbmR *mutant*

In order to investigate the possible biological implication of the *xbmR* gene for the pathogenicity of *Xcc*, we performed infection assays in grapefruits (*Citrus paradise* cv. *Macfadyen*). Each strain was inoculated on leaves of grapefruit by one of two different methods of infection: rubbing leaves gently with cotton swabs dipped in a suspension of bacteria or swabbing leaves previously injured with a needle to let the bacteria bypass stomatal entry and enter directly into the mesophyll. After 4 weeks, symptoms on leaves were observed. The *xbmR* mutant developed canker symptoms similar to the wild-type strain when the infection method involved injury to the leaf (Fig. 7A). However, when bacteria were swabbed on intact leaves, the *xbmR* mutant showed a significant reduction in the number of canker symptoms in comparison with the wild-type strain (Fig. 7B). Moreover, introduction of the *PA2567* gene into the *xbmR* mutant restored its ability to produce canker, when the methodology of infection was only swabbing (Fig. 7D). No alteration in the ability of infection was observed when the *wspR19* gene was introduced into the *xbmR* mutant (Fig. 7C).

Quantification of canker lesions in grapefruit leaves was done after 20 days of infection. The *xbmR* mutant was severely compromised in its ability to cause canker disease. The introduction of WspR19 did not restore virulence in *xbmR* mutant. In contrast, the introduction of PA2567 in the *xbmR* mutant led to the production of nearly the same number of cankers as for the wild-type strain (Fig. 7E).

Discussion

The findings in this paper add to our understanding of the factors that contribute to the ability of *Xcc* to form mature biofilms and to pathogenesis by establishing of role for XbmR (XAC3733) in these processes. To our knowledge, this is the first description of the role of this gene in chemotaxis and biofilm formation in the genus of *Xanthomonas*, although previous studies in *V. vulnificus* have shown that an NtrC family protein is associated with biofilm formation (Kim *et al*., 2007). In *Xcc*, the defects in chemotaxis and biofilm formation in the *xbmR* mutant strain could both be a result of the absence of the flagellar structure which is essential for motility in the chemotaxis assay and in the development of a mature biofilm (Malamud *et al*., 2011).

Mutation of *xbmR* had no effect on expression of *rpoN2*, but did influence *flgM*, *fliA*, *flhA*, *flhF*, *fleN*

and *fliC* expression. XbmR shares homology with the 'enhancer binding proteins' that form a complex with RpoN2 necessary for transcription of RpoN2-dependent promoters (Studholme and Dixon, 2003). Mutations in RpoN2 lead to loss of motility and virulence in *Xanthomonas* spp. (Tian *et al*., 2014). XbmR could possibly interact with RpoN2 to positively regulate the expression of flagellar genes.

Findings also suggest a role for the second messenger c-di-GMP in XbmR-mediated biofilm formation and flagellar biogenesis. A plausible mechanism by which XbmR modulates the c-di-GMP level in the cell is via the regulation of FliA and consequent effects on expression of genes encoding a subset of proteins involved in c-di-GMP metabolism. XbmR positively regulates the expression of XAC0350, which encodes an HD-GYP domain c-di-GMP phosphodiesterase. Work in a number of bacteria indicates that c-di-GMP can regulate flagellum-based motility at multiple levels, to include gene transcription, flagellar assembly and flagellar function (Wolfe and Visick, 2008). Accordingly, FliA of *E. coli* regulates the expression of the YhjH, an EAL domain c-di-GMP phosphodiesterase that is implicated in control of flagellar rotation (Claret *et al*., 2007; Boehm *et al*., 2010).

The expression of the PA2567 phosphodiesterase in the *xbmR* mutant of *Xcc* restored *fliC* expression, flagella synthesis and motility, but the expression of *fliA* in this strain did not return to the wild-type level. This is consistent with the suggestion that the alterations in the c-di-GMP are acting downstream of FliA in the flagellar synthesis hierarchy. However, the level of c-di-GMP was not significantly altered in the *xbmR* mutant compared with the wild type. This may suggest that XbmR influences a localized 'pool' of c-di-GMP, rather than the global level. In this scenario, the expression of *fliC* is positively regulated by *xbmR* (through FliA) but negatively regulated by c-di-GMP possibly generated by specific diguanylate cyclases; this latter action is normally reversed in the wild type by the HD-GYP domain protein XAC0350. Expression of *PA2567* can substitute for the loss of XAC0350, but expression of WspR19 swamps the system with massive c-di-GMP levels. The involvement of specific subsets of c-di-GMP diguanylate cyclases and/or phosphodiesterases in regulation of motility, virulence and biofilm formation has been suggested previously by systematic analyses in *P. fluorescens*, *Salmonella*, *E. coli* and *V. cholerae* (Boehm *et al*., 2010; Ahmad *et al*., 2011; Newell *et al*., 2011). These studies and others have led to the concept of 'microcompartmentalization' and localized pools of c-di-GMP (Hengge, 2009; Paul *et al*., 2010; Shikuma *et al*., 2012; Romling *et al*., 2013).

Expression of PA2567 in the *xbmR* mutant partially restored the biofilm structure whereas expression of WspR19 in wild type reduced biofilm formation (data not shown). These findings seem to contradict the generalization that high levels of c-di-GMP promote sessility and biofilm formation. One rationalization is that, under the conditions used, the absence of a functional flagellum necessary for adherence is of primary importance.

A number of other *Xanthomonas* species encode homologues of XbmR; these homologues include XerR from *X. campestris*. A defined role of XerR is in inhibition of *xccR*; *Xcc*R positively controls expression of proline iminopeptidase. The inhibitory action of XerR is reversed by plant extracts (activating phosphorylation) and plant extracts also activate *Xcc*R (Wang *et al*., 2011). Intriguingly, OryR, which is a homologue of *Xcc*R found in *X. oryzae* also responds to plant extracts and is implicated in positive control of motility and *fliC* expression (Gonzalez *et al*., 2013). A homologue of OryR (XAC2988) is found in *Xcc*. Although these homologies may suggest by extension that XbmR influences motility in *Xcc* by negative regulation of the activator XAC2988, this cannot *per se* account for the observation that the *xbmR* mutant has *reduced* motility and *fliC* expression.

Expression of the PA2567 phosphodiesterase activity restored virulence to the *xbmR* mutant. These observations suggest that motility and chemotaxis are important for bacterial penetration through stomatal pores, consistent with our previous observations that the absence of flagellin produces a slight reduction in *Xcc* pathogenicity to lemon leaves (Malamud *et al*., 2011). The possibility that XbmR regulates other phenotypes required for stomatal penetration should not be overlooked however.

Further investigation is now required to assign the exact mechanism of action of the XbmR protein in the regulation of flagellar genes and biofilm formation through fluctuations of c-di-GMP levels. These future studies will shed light on the regulatory pathways for biofilm development by *Xcc* and may provide the tools to attenuate its pathogenicity by controlling biofilm formation within the host environment.

Experimental procedures

Bacterial strains, plasmids and growth conditions

Xanthomonas strains were cultured at 28°C with shaking at 220 r.p.m. in PYM (Cadmus *et al*., 1976) or YMM (Sherwood, 1970). To examine biofilm development, bacteria were grown in YMM containing glucose (1% w/v) as the only carbon source (Rigano *et al*., 2007). *Escherichia coli* was grown at 37°C in Luria–Bertani medium (Sambrook *et al*., 1989). Bacterial growth was measured in a Spectronic 20 Genesys spectrophotometer (Thermo Electron Corp.) at 600 nm. When required, the antibiotics ampicillin (100 μg ml^{-1}), kanamycin (50 μg ml^{-1}), tetracycline, (5 μg ml^{-1}) and chloramphenicol (35 μg ml^{-1}) were added to the growth media. The tetracycline-resistant plasmids pLAFR6-PA2567 and

PME6032-*wspR19* were from Ryan and colleagues (2006) (Table S1).

Complementation of the xbmR *mutant*

To complement the *xbmR* mutant, a 1702 bp DNA fragment containing the entire open reading frame of *XAC3733* gene and a 350 bp sequence upstream of the start codon was polymerase chain reaction (PCR) amplified (Table S1, Fig. S1). The PCR product was cloned into a pGEMT-easy vector (Promega, Madison, WI, USA) and then digested with BamHI and KpnI. This fragment was ligated into pBBR1-MCS (Kovach *et al*., 1994). This construct was used to transform the *Xcc* mutant *ntrC* by electroporation as described previously (Malamud *et al*., 2011).

Motility assays

To evaluate the chemotactic ability of the *Xcc* strains, we measured their migration towards a chemical gradient generated by grapefruit leaves extracts. In order to prepare the extract, 20 g of young grapefruit leaves were washed with water three times and subsequently homogenized in 40 ml of distilled water. The solution was heated at 60°C and sterilized with 0.2 micron filter.

Chemotaxis was tested as described by de Weert and collagues (2002). Briefly, *Xcc* strains were grown in PYM medium and harvested in logarithmic phase. Cells were washed twice with YMM medium and resuspended in 12 ml of YMM to a final OD_{600} of 0.6. Finally, OD_{600} was adjusted to 0.4 with an aqueous solution of 1% hydroxypropylmethylcellulose (hypromellose) (Sigma-Aldrich, St. Louis, MO, USA). The resulting cell suspension was then poured into 60 mm-diameter Petri dishes creating a thin layer. Afterwards, 10 µl of extract of leaves was placed in the centre of each plate. After incubation from 30 min to 1 h at room temperature, the plates were analysed. The formation of a turbid zone around extracts was considered a positive chemotactic response (de Weert *et al*., 2002).

Swimming motility assays were carried out as described by Malamud and colleagues (2013).

Examination of flagella

Bacteria were grown overnight in PYM broth without glucose. Three biological replicates for each strain were evaluated. The samples were prepared by placing a bacterial suspension drop on a copper grid (400 mesh) covered by collodion for 5 min. Those bacteria attached to the grid were contrasted with 2% phosphotungstic acid for 40 s. The samples were examined with a transmission electron microscope JEM 1200 EX II (JEOL, Tokyo, Japan) at an operating voltage of 60 kV. Digital images were captured with a camera Erlangshen ES1000W, Model 785 (Gatan, Pleasanton, CA, USA).

Flagellum staining for subsequent observation by optical microscopy was performed as previously described (Kodaka *et al*., 1982). Briefly, the staining solution was prepared by mixing 10 parts of the mordant solution (2 g tannic acid, 10 ml 5% w/v phenol, 10 ml saturated aqueous of $AlKO_8S_2 12H2O$) with one part of the crystal violet solution (12% w/v of crystal violet in ethanol). A fresh colony was picked, resuspended in a drop of distilled water on a microscope slide and air dried. The sample was stained with 10 ml of the staining solution. Observations were made with Nikon ECLIPSE E600 optical microscope at 1000x (Nikon Instruments, Melville, NY, USA).

RNA extraction, cDNA synthesis and comparative qPCR

Total RNA for each strain was extracted from bacterial culture in mid-exponential growth phase using TRIzol (Gibco-BRL, Burlington, ON, Canada) following the manufacturer's instructions.

Reverse transcription was carried out using random primers and the Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT) (Promega, Madison, WI, USA).

Primers for quantitative detection (qRT-PCR) were designed using the system software (Applied Biosystems, Foster City, CA, USA) (Table S1). Reactions were performed using SybrGreen master mix (Roche, Mannheim, Germany) and qRT-PCR was performed with a Step One thermocycler (Applied Biosystems). Conditions for the qRT-PCR were as follows: 50°C for 2 min, initial denaturation at 95°C for 5 min, followed by 40 cycles of 10 s at 95°C and 30 s at 60°C.

The comparative cycle threshold method ($\Delta\Delta$Ct) was used to analyse data, as described by the manufacturer (Applied Biosystems). Gene expression levels obtained by qRT-PCR were normalized to 16S ribosomal gene expression. Data presented are averages of three independent experiments. Error bars represent the standard deviation of the samples.

In vitro analysis of biofilm formation by CLSM

For in vitro experiments, strains were grown at 28°C on PYM supplemented with the corresponding antibiotic. Cultures were diluted 1:1000 in YMM medium and aliquots of 500 µl were transferred to chambered coverglass slides containing a 1 mm thick borosilicate glass (Nunc, Wiesbaden, Germany). Strains were grown in the chambers for 4 days at 28°C until visualization (Malamud *et al*., 2011).

To allow visualization of biofilm structures, bacteria were stained with the BacLight LIVE/DEAD viability kit (Invitrogen, Paisley, UK). In brief, this kit consists in two fluorescent dyes: Syto-9t (S-9) and propidium iodide. The first of them stains viable bacteria whereas the second one is an indicator of the dead ones.

Biofilm formation was monitored with an inverted microscope Nikon Eclipse TE 2000-E2 CLSM (Nikon, Melville, NY, USA). Three-dimensional images were generated with the Image J1.4 software (Wayne Rasband, National Institutes of Health, USA) (http://rsbweb.nih.gov/ij/download.html) and COMSTAT analysis was performed using the Image Processing Toolbox as previously described Heydorn and colleagues (2000).

Cyclic di-GMP measurement

Cyclic di-GMP was quantified as described previously Ryan and colleagues (2009) in bacterial strains grown to an OD at 600 nm of 0.8 in NYGB medium.

Expression of XAC0350 HD-GYP domain X. campestris *rpfG mutant*

DNA fragments encoding the HD-GYP domain from XAC0350 were amplified by PCR by using chromosomal DNA of *Xcc*, as template with primers XAC0350F2 and XAC0350R (Table S1). These amplified fragments were cloned into the TOPO (Invitrogen) vector. The appropriate DNA fragments were excised from these constructs using EcoR1 and HindIII and ligated into pLAFR3 (Staskawicz *et al*., 1987) that had been cut with the same enzymes so that expression is under control of the *lac* promoter of the vector. Constructs were introduced into *X. campestris rpfG* mutant by triparental mating using the helper plasmid pRK2073.

Endoglucanase activity determination

Endoglucanase activities in culture supernatants of bacterial strains grown to an OD at 600 nm 2.0 in NYGB medium were determined as described by Ryan and colleagues (2006).

Plant growth conditions and infection

Citrus paradise cv. *Macfadyen* was used as the host plant for *Xcc*. All plant inoculations involved at least three leaves from each plant and three plants were inoculated with each strain of *Xcc* tested. Plants were kept in a room with controlled temperature and a photoperiod of 16 h. Bacterial suspensions were grown in PYM medium with the appropriate antibiotics and diluted in sterile distilled water at final concentration of 1×10^6 colony-forming unit (cfu) ml^{-1}. Two methods of infection were applied: swabbing on leaves previously injured with a needle or swabbing leaves without injuries. Symptoms were observed after 4 weeks of infection.

Cankers from 20 inoculated leaves were quantified and their areas calculated using the ImageJ 1.4 sofware.

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References

Ahmad, I., Lamprokostopoulou, A., Le Guyon, S., Streck, E., Barthel, M., Peters, V., *et al*. (2011) Complex c-di-GMP signaling networks mediate transition between virulence properties and biofilm formation in *Salmonella enterica* serovar *Typhimurium*. *PLoS ONE* **6:** e28351.

Andrade, M.O., Alegria, M.C., Guzzo, C.R., Docena, C., Rosa, M.C., Ramos, C.H., and Farah, C.S. (2006) The HD-GYP domain of RpfG mediates a direct linkage between the Rpf quorum-sensing pathway and a subset of diguanylate cyclase proteins in the phytopathogen *Xanthomonas axonopodis* pv citri. *Mol Microbiol* **62:** 537–551.

Bellini, D., Caly, D.L., McCarthy, Y., Bumann, M., An, S.Q., Dow, J.M., *et al*. (2014) Crystal structure of an HD-GYP domain cyclic-di-GMP phosphodiesterase reveals an enzyme with a novel trinuclear catalytic iron centre. *Mol Microbiol* **91:** 26–38.

Boehm, A., Kaiser, M., Li, H., Spangler, C., Kasper, C.A., Ackermann, M., *et al*. (2010) Second messenger-mediated adjustment of bacterial swimming velocity. *Cell* **141:** 107–116.

Brunings, A.M., and Gabriel, D.W. (2003) *Xanthomonas citri*: breaking the surface. *Mol Plant Pathol* **4:** 141–157.

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.

Claret, L., Miquel, S., Vieille, N., Ryjenkov, D.A., Gomelsky, M., and Darfeuille-Michaud, A. (2007) The flagellar sigma factor FliA regulates adhesion and invasion of Crohn disease-associated *Escherichia coli* via a cyclic dimeric GMP-dependent pathway. *J Biol Chem* **282:** 33275–33283.

De Carlo, S., Chen, B., Hoover, T.R., Kondrashkina, E., Nogales, E., and Nixon, B.T. (2006) The structural basis for regulated assembly and function of the transcriptional activator NtrC. *Genes Dev* **20:** 1485–1495.

Gonzalez, J.F., Myers, M.P., and Venturi, V. (2013) The interkingdom solo OryR regulator of *Xanthomonas oryzae* is important for motility. *Mol Plant Pathol* **14:** 211–221.

Guttenplan, S.B., and Kearns, D.B. (2013) Regulation of flagellar motility during biofilm formation. *FEMS Microbiol Rev* **37:** 849–871.

Hengge, R. (2009) Principles of c-di-GMP signalling in bacteria. *Nat Rev Microbiol* **7:** 263–273.

Hervas, A.B., Canosa, I., Little, R., Dixon, R., and Santero, E. (2009) NtrC-dependent regulatory network for nitrogen assimilation in *Pseudomonas putida*. *J Bacteriol* **191:** 6123–6135.

Heydorn, A., Nielsen, A.T., Hentzer, M., Sternberg, C., Givskov, M., Ersboll, B.K., and Molin, S. (2000) Quantification of biofilm structures by the novel computer program COMSTAT. *Microbiology* **146** (Part 10): 2395–2407.

Hirschman, J., Wong, P.K., Sei, K., Keener, J., and Kustu, S. (1985) Products of nitrogen regulatory genes *ntrA* and *ntrC* of enteric bacteria activate *glnA* transcription in vitro: evidence that the *ntrA* product is a sigma factor. *Proc Natl Acad Sci USA* **82:** 7525–7529.

Kim, D., Chung, J., Hyun, H., Lee, C., Lee, K., and Cho, K. (2009a) Operon required for fruiting body development in *Myxococcus xanthus*. *J Microbiol Biotechnol* **19:** 1288–1294.

Kim, H.S., Lee, M.A., Chun, S.J., Park, S.J., and Lee, K.H. (2007) Role of NtrC in biofilm formation via controlling expression of the gene encoding an ADP-glycero-manno-heptose-6-epimerase in the pathogenic bacterium, *Vibrio vulnificus*. *Mol Microbiol* **63:** 559–574.

Kim, H.S., Park, S.J., and Lee, K.H. (2009b) Role of NtrC-regulated exopolysaccharides in the biofilm formation and

pathogenic interaction of *Vibrio vulnificus*. *Mol Microbiol* **74:** 436–453.
Kim, Y.K., and McCarter, L.L. (2007) ScrG, a GGDEF-EAL protein, participates in regulating swarming and sticking in *Vibrio parahaemolyticus*. *J Bacteriol* **189:** 4094–4107.
Kodaka, H., Armfield, A.Y., Lombard, G.L., and Dowell, V.R., Jr (1982) Practical procedure for demonstrating bacterial flagella. *J Clin Microbiol* **16:** 948–952.
Kovach, M.E., Phillips, R.W., Elzer, P.H., Roop, R.M., II, and Peterson, K.M. (1994) pBBR1MCS: a broad-host-range cloning vector. *Biotechniques* **16:** 800–802.
Kroos, L. (2007) The *Bacillus* and *Myxococcus* developmental networks and their transcriptional regulators. *Annu Rev Genet* **41:** 13–39.
Kustu, S., Santero, E., Keener, J., Popham, D., and Weiss, D. (1989) Expression of sigma 54 (*ntrA*)-dependent genes is probably united by a common mechanism. *Microbiol Rev* **53:** 367–376.
Lemon, K.P., Higgins, D.E., and Kolter, R. (2007) Flagellar motility is critical for *Listeria monocytogenes* biofilm formation. *J Bacteriol* **189:** 4418–4424.
Li, J., and Wang, N. (2011) Genome-wide mutagenesis of *Xanthomonas axonopodis* pv. *citri* reveals novel genetic determinants and regulation mechanisms of biofilm formation. *PLoS ONE* **6:** e21804.
Malamud, F., Torres, P.S., Roeschlin, R., Rigano, L.A., Enrique, R., Bonomi, H.R., *et al*. (2011) The *Xanthomonas axonopodis* pv. *citri* flagellum is required for mature biofilm and canker development. *Microbiology* **157:** 819–829.
Malamud, F., Conforte, V.P., Rigano, L.A., Castagnaro, A.P., Marano, M.R., Morais do Amaral, A., and Vojnov, A.A. (2012) HrpM is involved in glucan biosynthesis, biofilm formation and pathogenicity in *Xanthomonas citri* ssp. *citri*. *Mol Plant Pathol* **13:** 1010–1018.
Malamud, F., Homem, R.A., Conforte, V.P., Yaryura, P.M., Castagnaro, A.P., Marano, M.R., *et al*. (2013) Identification and characterization of biofilm formation-defective mutants of *Xanthomonas citri* subsp. *citri*. *Microbiology* **159:** 1911–1919.
Murga, R., Stewart, P.S., and Daly, D. (1995) Quantitative analysis of biofilm thickness variability. *Biotechnol Bioeng* **45:** 503–510.
Newell, P.D., Yoshioka, S., Hvorecny, K.L., Monds, R.D., and O'Toole, G.A. (2011) Systematic analysis of diguanylate cyclases that promote biofilm formation by *Pseudomonas fluorescens* Pf0-1. *J Bacteriol* **193:** 4685–4698.
O'Connell, A., An, S.Q., McCarthy, Y., Schulte, F., Niehaus, K., He, Y.Q., *et al*. (2013) Proteomics analysis of the regulatory role of Rpf/DSF cell-to-cell signaling system in the virulence of *Xanthomonas campestris*. *Mol Plant Microbe Interact* **26:** 1131–1137.
O'Toole, G.A., and Kolter, R. (1998) Initiation of biofilm formation in *Pseudomonas fluorescens* WCS365 proceeds via multiple, convergent signalling pathways: a genetic analysis. *Mol Microbiol* **28:** 449–461.
Paul, K., Nieto, V., Carlquist, W.C., Blair, D.F., and Harshey, R.M. (2010) The c-di-GMP binding protein YcgR controls flagellar motor direction and speed to affect chemotaxis by a 'backstop brake' mechanism. *Mol Cell* **38:** 128–139.
Rigano, L.A., Siciliano, F., Enrique, R., Sendin, L., Filippone, P., Torres, P.S., *et al*. (2007) Biofilm formation, epiphytic fitness, and canker development in *Xanthomonas axonopodis* pv. *citri*. *Mol Plant Microbe Interact* **20:** 1222–1230.
Romling, U. (2012) Cyclic di-GMP, an established secondary messenger still speeding up. *Environ Microbiol* **14:** 1817–1829.
Romling, U., Galperin, M.Y., and Gomelsky, M. (2013) Cyclic di-GMP: the first 25 years of a universal bacterial second messenger. *Microbiol Mol Biol Rev* **77:** 1–52.
Ruffing, A.M., and Chen, R.R. (2012) Transcriptome profiling of a curdlan-producing *Agrobacterium* reveals conserved regulatory mechanisms of exopolysaccharide biosynthesis. *Microb Cell Fact* **11:** 17.
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., Lucey, J., O'Donovan, K., McCarthy, Y., Yang, L., Tolker-Nielsen, T., and Dow, J.M. (2009) HD-GYP domain proteins regulate biofilm formation and virulence in *Pseudomonas aeruginosa*. *Environ Microbiol* **11:** 1126–1136.
Sambrook, J., Fritsch, E., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor, NY, USA: Cold Spring Harbor Laboratory.
Sherwood, M. (1970) Improved synthetic medium for the growth of *Rhizobium*. *J Appl Bacteriol* **33:** 708–713.
Shikuma, N.J., Fong, J.C., and Yildiz, F.H. (2012) Cellular levels and binding of c-di-GMP control subcellular localization and activity of the *Vibrio cholerae* transcriptional regulator VpsT. *PLoS Pathog* **8:** e1002719.
Siciliano, F., Torres, P.S., Sendin, L., Bermejo, C., Filippone, P., Vellice, G., *et al*. (2006) Analysis of the molecular basis of *Xanthomonas axonopodis* pv. *citri* pathogenesis in *Citrus limon*. *Electron J Biotechnol* **9:** 200–204.
Sondermann, H., Shikuma, N.J., and Yildiz, F.H. (2012) You've come a long way: c-di-GMP signaling. *Curr Opin Microbiol* **15:** 140–146.
Staskawicz, B., Dahlbeck, D., Keen, N., and Napoli, C. (1987) Molecular characterization of cloned avirulence genes from race 0 and race 1 of *Pseudomonas syringae* pv. *glycinea*. *J Bacteriol* **169:** 5789–5794.
Studholme, D.J., and Dixon, R. (2003) Domain architectures of sigma54-dependent transcriptional activators. *J Bacteriol* **185:** 1757–1767.
Tian, F., Yu, C., Li, H., Wu, X., Li, B., Chen, H., *et al*. (2014) Alternative sigma factor RpoN2 is required for flagellar motility and full virulence of *Xanthomonas oryzae* pv. *oryzae*. *Microbiol Res*. doi.org/10.1016/j.micres.2014.07.002.
Wang, L., Zhang, L., Geng, Y., Xi, W., Fang, R., and Jia, Y. (2011) XerR, a negative regulator of *Xcc*R in *Xanthomonas campestris* pv. *campestris*, relieves its repressor function in planta. *Cell Res* **21:** 1131–1142.
de Weert, S., Vermeiren, H., Mulders, I.H., Kuiper, I., Hendrickx, N., Bloemberg, G.V., *et al*. (2002) Flagella-driven chemotaxis towards exudate components is an important trait for tomato root colonization by *Pseudomonas fluorescens*. *Mol Plant Microbe Interact* **15:** 1173–1180.

Wolfe, A.J., and Visick, K.L. (2008) Get the message out: cyclic-Di-GMP regulates multiple levels of flagellum-based motility. *J Bacteriol* **190:** 463–475.

Yang, T.C., Leu, Y.W., Chang-Chien, H.C., and Hu, R.M. (2009) Flagellar biogenesis of *Xanthomonas campestris* requires the alternative sigma factors RpoN2 and FliA and is temporally regulated by FlhA, FlhB, and FlgM. *J Bacteriol* **191:** 2266–2275.

Yu, X., Lund, S.P., Scott, R.A., Greenwald, J.W., Records, A.H., Nettleton, D., *et al*. (2013) Transcriptional responses of *Pseudomonas syringae* to growth in epiphytic versus apoplastic leaf sites. *Proc Natl Acad Sci USA* **110:** E425–E434.

Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1. Insertion site Tn5.

Fig. S2. Chemotaxis assays of the *Xcc* 306, *Xcc* 306 (PA2667) and *Xcc* 306 (wspR19) strains.

Fig. S3. Swimming motility assays of the *Xcc* 306, *xbmR* and *c-xmbR* strains.

Table S1. Strains, plasmids and primers used in this work.

Table S2. Putative FliA-dependent promoters in *Xcc*306.

Table S3. Assessment of XAC0350 as a potential c-di-GMP phosphodiesterase. The activity of XAC0350 as a potential c-di-GMP phosphodiesterase was assessed by expression of the HD-GYP domain in the *rpfG* mutant of *X. campestris*. The high level of c-di-GMP in this strain leads to repression of extracellular endoglucanase synthesis; this effect is reversed by expression of active c-di-GMP phosphodiesterases (Ryan *et al*., 2006). All data represent the mean ± standard deviation of triplicate measurements; different letters indicate significant differences ($P < 0.01$).

Video Clip S1. WT motility.

Video Clip S2. *xbmR* motility .