

The *Xanthomonas axonopodis* pv. *citri* flagellum is required for mature biofilm and canker development

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Xanthomonas axonopodis pv. *citri* (*Xac*) is the causative agent of citrus canker. This bacterium develops a characteristic biofilm on both biotic and abiotic surfaces. To evaluate the participation of the single flagellum of *Xac* in biofilm formation, mutants in the *fliC* (flagellin) and the *flgE* (hook) genes were generated. Swimming motility, assessed on 0.25 % agar plates, was markedly reduced in *fliC* and *flgE* mutants. However, the *fliC* and *flgE* mutants exhibited a flagellar-independent surface translocation on 0.5 % agar plates. Mutation of either the *rpfF* or the *rpfC* gene, which both encode proteins involved in cell–cell signalling mediated by diffusible signal factor (DSF), led to a reduction in both flagellar-dependent and flagellar-independent surface translocation, indicating a regulatory role for DSF in both types of motility. Confocal laser scanning microscopy of biofilms produced in static culture demonstrated that the flagellum is also involved in the formation of mushroom-shaped structures and water channels, and in the dispersion of biofilms. The presence of the flagellum was required for mature biofilm development on lemon leaf surfaces. The absence of flagellin produced a slight reduction in *Xac* pathogenicity and this reduction was more severe when the complete flagellum structure was absent.

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INTRODUCTION

Biofilms are bacterial communities in which cells are embedded in an extracellular polysaccharide (EPS) matrix that can be attached to a surface (Branda *et al.*, 2005; Southey-Pillig *et al.*, 2005). Life in these communities provides protection to the organisms from deleterious conditions (Davey & O'Toole, 2000), and biofilm formation is considered to be important for the disease cycle of bacterial pathogens of both animals and plants. We are interested in understanding the role of biofilm formation in the development of canker disease by *Xanthomonas axonopodis* pv. *citri* (*Xac*), one of the most devastating

diseases of citrus species. *Xac* is a foliar pathogen that enters the plant leaves through stomata and can infect both fruits and leaves (Brunings & Gabriel, 2003). Confocal laser scanning microscopy (CLSM) of citrus cankers, using *Xac* bacteria expressing green fluorescent protein (GFP), showed the occurrence of structured arrangements of cells (Rigano *et al.*, 2007b). Our aim here was to identify bacterial factors involved in the development of such three-dimensional structures.

The development of bacterial biofilms is generally a multistep process, which is initiated when the bacteria reach a surface. First, the bacteria attach to a surface reversibly, where they can move freely across it until they become immobilized (Stoodley *et al.*, 2002). To achieve a mature biofilm, new individuals appear in the community either by recruitment from planktonic bacteria or through replication of cells already present in the biofilm. Finally, some grouped cells disperse to develop new structures elsewhere (Heydorn *et al.*, 2000; Tolker-Nielsen *et al.*,

Abbreviations: CLSM, confocal laser scanning microscopy; CV, crystal violet; DSF, diffusible signal factor; EPS, extracellular polysaccharide; *Xac*, *Xanthomonas axonopodis* pv. *citri*; *Xcc*, *Xanthomonas campestris* pv. *campestris*.

Two supplementary figures are available with the online version of this paper.

2000). When the bacterial population reaches a certain density, the biofilm begins to mature through the production of an extracellular matrix which contributes greatly to the final architecture of the community (Branda *et al.*, 2005).

Flagella have been implicated in surface attachment in several bacteria (Lemon *et al.*, 2007; O'Toole & Kolter, 1998a, b). For instance, flagella are necessary for swarming motility in *Pseudomonas aeruginosa*, which in turn is important in determining the final structure of the biofilm (Merritt *et al.*, 2007). Similarly, *Escherichia coli* is not able to form an organized structure when flagella are lost. It has been proposed that *E. coli* flagella serve to overcome surface repulsion (Van Houdt & Michiels, 2005) and also allow attached cells to migrate along the abiotic surface to facilitate biofilm expansion (Pratt & Kolter, 1998). However, flagella are important but not essential for surface attachment in *Vibrio cholerae* El Tor (Watnick & Kolter, 1999).

Previous work from our laboratory has established that synthesis of the EPS xanthan contributes to the formation of structured biofilms in *Xac* and that both synthesis of xanthan and cell–cell signalling involving diffusible signal factor (DSF) contribute to structured biofilm formation in the related *Xanthomonas campestris* pv. *campestris* (*Xcc*) (Rigano *et al.*, 2007a; Torres *et al.*, 2007). Synthesis and perception of DSF require proteins coded in the *rpf* cluster (for regulation of pathogenicity factors). RpfF directs DSF synthesis whereas RpfC is involved in DSF perception (Barber *et al.*, 1997; Slater *et al.*, 2000). Mutants in the *rpfF* (DSF-minus) and *rpfC* (DSF overproducer) genes in *Xcc* can only form unstructured arrangements of bacteria (Torres *et al.*, 2007). Synthesis of xanthan is directed by genes from the *gum* operon (da Silva *et al.*, 2002), which is highly conserved in *Xanthomonas* spp. Disruption of the first gene, *gumB*, leads to complete loss of xanthan production (Vojnov *et al.*, 1998); strains of *Xac* or *Xcc* with mutations in *gumB* cannot form structured biofilms (Rigano *et al.*, 2007b; Torres *et al.*, 2007). Beyond DSF signalling and xanthan production, relatively little is known about factors influencing biofilm formation in *Xanthomonas* spp. and the role that the flagellum plays in this process.

Both *Xcc* and *Xac* bear a single polar flagellum. Flagellar gene clusters encode all the structural proteins of the flagellum in *Xac* (da Silva *et al.*, 2002). In addition, it has been shown that several genes encoding auxiliary protein subunits are involved in regulation of the flagellar assembly (Khater *et al.*, 2007). As in many other bacterial species, FliC and FlgE proteins are the flagellin (Vonderviszt *et al.*, 1998) and the hook components (Aizawa, 1996), respectively, of the flagellar structure. This structure is formed by a thin filament that protrudes from the cell body bound to a basal body that includes a rotor and a stator (Chevance & Hughes, 2008).

Here we address the role of the single flagellum in biofilm formation in *Xac*. Our initial approach was to test the

effects of mutations in *fliC* and *flgE* on motility and biofilm structure. Our results indicate that the flagellum and flagellum-dependent motility are important for initial adherence to surfaces, for the development of a mature structured biofilm and for biofilm dispersion. Unexpectedly, our observations reveal a second type of motility that is flagellum-independent and EPS-dependent. Both types of motility are regulated by the DSF signalling system. Thus, these findings indicate possible additional roles for cell–cell signalling and EPS in the biofilm formation process.

METHODS

Bacterial strains. *Xanthomonas* strains were cultured at 28 °C with shaking in PYM (Cadmus *et al.*, 1976) or in Y minimal medium (YMM) (Sherwood, 1970). To examine biofilm development, bacteria were grown in YMM containing 1 % (w/v) glucose as the carbon source (Rigano *et al.*, 2007b). *E. 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) at 600 nm. When required, the antibiotics ampicillin (100 µg ml⁻¹), kanamycin (50 µg ml⁻¹), spectinomycin (100 µg ml⁻¹), tetracycline (10 µg ml⁻¹) or gentamicin (20 µg ml⁻¹) were added to the growth media.

Construction of *fliC* and *flgE* mutants. Molecular techniques and extraction of genomic DNA from *Xac* were performed according to Rigano *et al.* (2007b). To obtain the *Xac*Δ*fliC* mutant strain (*fliC*), the *fliC* gene was partially deleted. First, two fragments of 408 and 484 bp were amplified from the region encoding the wild-type *Xac* *fliD* and *fliC* genes by using primers *flic1/flic2* and *flic3/flic4*, respectively (Table 1). The fragments were digested with restriction enzyme *Xba*I, purified and ligated to one another. A PCR was then performed using this ligation as template and primers *flic1/flic4*. The PCR product was resolved by 0.8 % agarose gel electrophoresis and a fragment of 892 bp was excised and purified from the gel using the Wizard SV Gel and PCR Clean-Up System (Promega). The product was cloned into pGEM-T easy (Promega) and subcloned into the suicide vector *pk18mobsacB* (Katzen *et al.*, 1999), producing the *pK*-Δ*fliC* construct.

The *Xac* *flgE*::Ω strain (*flgE*) was constructed by allelic exchange. Two regions, upstream and downstream from *flgE*, were amplified using *flge1/flge2* (546 bp) and *flge3/flge4* (547 bp) primers, respectively (Table 1). The two fragments were digested with *Hind*III, purified and ligated to one another, and a PCR was then performed using this ligation as template and primers *flge1/flge4*. The PCR product of 1093 bp was resolved and purified as described above. The product was cloned into pGEM-T easy and the 2 kb *Sm*^r/*Spc*^r cassette (Ω) was inserted in the *Hind*III restriction site. This construct was subcloned into *pk18mobsacB*, giving *pK*-*flgE*::Ω.

The two constructs, *pK*-Δ*fliC* and *pK*-*flgE*::Ω, were used to transform the wild-type strain by electroporation (do Amaral *et al.*, 2005). Transformed bacteria were selected on PYM agar medium supplemented with kanamycin (Müller *et al.*, 1993). Sucrose-sensitive clones were grown in the absence of antibiotics and double recombination events were selected on PYM agar plates supplemented with 5 % sucrose and spectinomycin in the case of the *flgE* mutant. Km^s/sucrose^r candidates were screened by PCR followed by sequencing of the amplified fragments. Isolated mutants were confirmed by flagellum staining as described by Kearns & Losick (2003) and motility was further analysed.

For genetic complementation of the *fliC* mutant, the entire gene and 245 bp from the upstream region were amplified by PCR using

Table 1. Strains, plasmids and primers used in this study

Strain, plasmid or primer	Relevant genotype or sequence (5'–3')	Source or reference
Strains		
<i>Xac</i>	Wild-type, Ap ^r	Rigano <i>et al.</i> (2007b)
<i>Xac</i> GFP	<i>Xac</i> , Km ^r Ap ^r	This study
<i>fliC</i>	<i>Xac</i> Δ <i>fliC</i> , Ap ^r	This study
<i>flgE</i>	<i>Xac flgE::Ω</i> , Ap ^r Sp ^r	This study
<i>fliC</i> -GFP	<i>Xac</i> Δ <i>fliC</i> , Ap ^r Km ^r	This study
<i>flgE</i> -GFP	<i>Xac flgE::Ω</i> , Ap ^r Ap ^r	This study
<i>c-fliC</i>	<i>Xac</i> Δ <i>fliC</i> , Ap ^r Tc ^r	This study
<i>c-flgE</i>	<i>Xac flgE::Ω</i> , Ap ^r Sp ^r Tc ^r	This study
<i>rpjF</i>	<i>Xac</i> Δ <i>rpjF</i> , Km ^r Ap ^r	Siciliano <i>et al.</i> (2006)
<i>rpjC</i>	<i>Xac</i> Δ <i>rpjC</i> , Km ^r Ap ^r	Siciliano <i>et al.</i> (2006)
<i>gumB</i>	<i>Xac</i> Δ <i>gumB</i> , Km ^r Ap ^r	Rigano <i>et al.</i> (2007b)
<i>E. coli</i> DH5α	<i>hsdR recA lacZYA φ80 lacZΔM15</i>	Gibco-BRL
Plasmids		
pGem-Teasy	Ap ^r , <i>lacZ</i>	Promega
Pk mobsacB	Km ^r , <i>sacB mob lacZ</i>	Katzen <i>et al.</i> (1999)
pBBR2-GFP	Km ^r	Rigano <i>et al.</i> (2007b)
pLAFR 3	Tc ^r	Staskawicz <i>et al.</i> (1987)
pK-Δ <i>fliC</i>	Km ^r	This study
pK- <i>flgE::Ω</i>	Km ^r Sp ^r	This study
pLAFR- <i>fliC</i>	Tc ^r	This study
pLAFR- <i>flgE</i>	Tc ^r	This study
Primers		
Flic1	GCGAATTTCGCCTTGTGATGCGTGCCTG†	This study
Flic2	GCTCTAGATTCCGCAGAACGTGCTGAGC‡	This study
Flic3	GCTCTAGAGAGATAACCGTCGTTGGCGTT‡	This study
Flic4	GCGGATCCCAGGCGGACGGAGTTTATTT§	This study
Flge1	GGGAATCGCAAAAGCGGGAT	This study
Flge2	AAGCTTGCAGCTCAACGTCCTGGCT	This study
Flge3	AAGCTTCGTAGACATTGATGCCGCCG	This study
Flge4	CGCAGCCAATGCCGATCTGA	This study
c-Flic-sense	GCGAATTCCATTGCTGCGGCAGGTAAC†	This study
c-Flge-sense	GCGAATTTCGATGATCGTTTGTAGTGACCTGGTCC†	This study
c-Flge-antisense	GCGGATCCCCTCACCAACGGAACACTCCACAT§	This study
RT-flic sense	CAGCTTGGTGCCGTTGAAGTT	This study
RT-flic antisense	TGAACGCTCAGCGGAACCTCA	This study
RT-gumb sense	AAACACGATGACATTGCCGC	This study
RT-gumb antisense	GCCATATTTTCGTTGCCGCTC	This study
RT-16S sense	TGGTAGTCCACGCCCTAAACG	This study
RT-16S antisense	CTGGAAAGTTCGTTGGATGTC	This study

*Km^r, Ap^r, Gm^r, Sp^r and Tc^r indicate kanamycin, ampicillin, gentamicin, spectinomycin and tetracycline resistance, respectively. Sites for restriction enzymes are underlined: †*EcoRI*, ‡*XbaI*, §*BamHI*, ||*HindIII*.

primers c-Flic-sense and Flic4 (Table 1). The amplified fragment was cloned into pLAFR3 (Staskawicz *et al.*, 1987) to obtain pLAFR-*fliC*. Similarly, the *flgE* gene was amplified using primers c-Flge-sense and c-Flge-antisense (Table 1); the amplicon was cloned into pLAFR3 to obtain pLAFR-*flgE*. Both constructs were confirmed by DNA sequencing and electroporated into *fliC* and *flgE* mutant strains.

Bacterial adhesion and biofilm quantification. The crystal violet (CV) technique was used to quantify biofilm development of the different strains on an abiotic surface (O'Toole & Kolter, 1998b). Bacterial strains were grown overnight in PYM nutrient medium and then inoculated into YMM to a final OD₆₀₀ of 0.1. Aliquots of 150 μl were used to fill the wells of a 96-well polystyrene plate and incubated

at 28 °C for 24, 48 or 72 h. To confirm similar bacterial growth, the OD₆₀₀ was measured before the adhesion assay was performed.

To analyse the capacity of the strains to adhere to the abiotic surface, bacteria from an overnight culture growth in YMM at 28 °C and 200 r.p.m. were used. Aliquots of 150 μl were dispensed into the wells of a 96-well plate and bacteria were incubated as previously described for 1, 3, 6 and 24 h. The OD₆₀₀ and cell attachment were measured as described above.

For quantification of biofilm development and adhesion of cells to an abiotic surface, the medium was gently removed using a pipette, and the 96-well plate was washed using 0.9% NaCl and stained with 0.1% CV solution. After 30 min incubation the unbound CV stain was

removed and the wells were washed with distilled water. The CV in each well was solubilized by adding 150 μ l 70% ethanol and was quantified by absorbance at 570 nm.

The adhesion value was normalized according to the number of cells. This value was termed relative adhesion (A_{570}/OD_{600}).

In vitro and in vivo analysis of biofilm by CLSM. For *in vitro* experiments each strain expressing the autofluorescent protein GFP was grown at 28 °C on PYM supplemented with kanamycin. The OD_{600} was adjusted to 0.004 in YMM. Aliquots of 500 μ l were transferred to chambered coverglass slides containing a 1 μ m thick borosilicate glass (no. 155411; Lab-Tek, Nunc), as described by Russo *et al.* (2006).

Biofilm formation was monitored with a Zeiss LSM 510/Axiocvert 100M confocal laser scanning microscope at 1, 2, 3, 4 and 5 days post-inoculation (days p.i.) of the bacteria on the chambered coverglass slides. The assay was performed in triplicate and three-dimensional images were generated by the program Zeiss LSM Image Browser, version 3.2.0.

For *in vivo* studies, leaves of lemon (*Citrus limon*) were infected by spraying with a 1×10^6 c.f.u. ml⁻¹ suspension of GFP-labelled wild-type *Xac* or the *fliC* or *flgE* mutants. After 6 days of incubation, areas of approximately 1 cm² were cut from the leaves and mounted on the adaxial leaf surface under glass coverslips. The samples were observed with a Nikon C1 confocal laser scanning microscope. The simulated images obtained were analysed with the program Nikon EZ-C1. Assays were performed in triplicate.

Quantification of biofilm structures. Quantifications of biofilm volume and thickness of three-dimensional CLSM biofilm image stacks were performed by using the COMSTAT program (Heydorn *et al.*, 2000). For comparative analysis of the wild-type, *fliC* and *flgE* mutant strains, three independent biofilm experiments were performed. In

each round, seven image stacks were acquired at the following time points: 1, 2, 3, 4 and 5 days p.i.

RNA purification and RT-PCR. Total RNA of *Xac* cells was isolated by using an RBC kit, according to the manufacturer's instructions (RBCBioscience). The RNA was treated with RNase-free DNase (Promega) and the integrity of the nucleic acid was checked by agarose gel electrophoresis. Total RNA (1 μ g) was used to synthesize the cDNA using MMLV-RT (Promega) and the oligonucleotide dN6. Expression of the *gumB*, *fliC* and 16S genes was determined by RT-PCR using the primers listed in Table 1.

The density corresponding to the RT-PCR bands was quantified by using ImageJ 1.41 software from the National Institutes of Health (<http://rsbweb.nih.gov/ij/download.html>). Each gene was normalized using 16S rRNA as the housekeeping gene. The ratio between normalized *fliC* or *gumB* in *rpfC* and *rpfF* mutants and in wild-type *Xac* was determined. Data are expressed as the mean \pm SEM ($n=3$).

Motility assays. Motility assays were performed as described by Rashid & Kornberg (2000). Briefly, bacteria were grown overnight in PYM medium, then 3 μ l of bacterial cultures with normalized OD_{600} were used to inoculate plates of 0.25% agar NYGB medium (Barber *et al.*, 1997) (swimming) and 0.5% agar PYM. After 72 h, motility was assessed qualitatively by examining the circular halo formed by the growing bacterial cells. The assays were performed in triplicate.

Flagellar staining with CV. Flagellar staining was done based on a modification of the method described by Mayfield & Inniss (1977). The staining solution was prepared by mixing 10 vols solution A (2 g tannic acid, 10 ml 5% phenol, 10 ml $AlK_2(SO_4)_3 \cdot 12H_2O$) with 1 vol. 12% (w/v) CV in ethanol. Then 3 μ l of bacterial culture grown overnight in PYM (OD_{600} 2) was placed over a coverglass with a coverslip and 10 μ l of the solution was used. Observations were made with an optical microscope (Carl Zeiss).

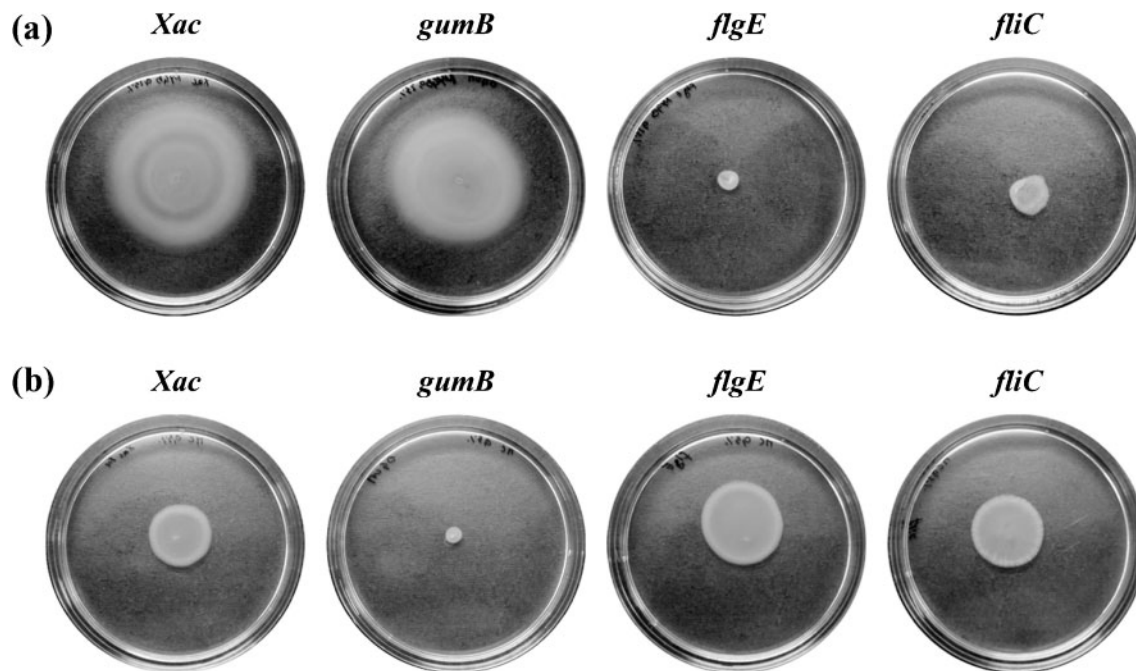


Fig. 1. Effects of mutation of the *fliC*, *flgE* and *gumB* genes on motility in *Xac*. (a) Flagellum-dependent swimming motility on 0.25% NYGB agar plates. (b) Motility on 0.5% PYM agar plates with 1% glucose.

Plant material and inoculations. *Citrus limon* 'Eureka' was used as the host plant for *Xac*. Plants were kept in a controlled temperature room at 20–25 °C and with a 16 h photoperiod. Bacteria were grown in PYM with the appropriate antibiotics. Bacterial suspensions were diluted in 10 mM MgCl₂ to a final concentration of 1×10^4 , 1×10^5 or 1×10^6 c.f.u. ml⁻¹ and were inoculated by spraying as previously described (Rigano *et al.*, 2007b). Bacterial growth in the host plant was quantified as previously described (Rigano *et al.*, 2007b), using 1×10^6 c.f.u. ml⁻¹ to spray the leaves. Statistical significance of differences between bacterial populations was determined by Student's unpaired two-tailed *t*-test. *P*-values <0.05 were considered statistically relevant. Cankers from 20 inoculated leaves were quantified and their areas were calculated using the program ImageJ version 1.41.

RESULTS

FliC and FlgE are required for swimming motility

The genomic arrangement near the *fliC* and *flgE* genes in *Xac* is shown in Supplementary Fig. S1(a), available with the online version of this paper. *Xac* strains with either a *fliC* deletion or a disruption in the *flgE* gene with a spectinomycin resistance cassette were created as described

in Methods (Supplementary Fig. S1b). The mutations were confirmed by PCR (Supplementary Fig. S1c) and DNA sequence analysis (data not shown). The absence of the flagellar structure in these mutants was confirmed by CV staining (Kearns & Losick, 2003). A typical single polar flagellum was observed in wild-type *Xac*, but was absent in the two mutants (Supplementary Fig. S1d). This observation confirms the expected and fundamental role for FliC and FlgE in the morphogenesis of the flagellum in *Xac*.

The ability of the different *Xac* strains to swim in low-percentage agar was assayed to evaluate the role of the flagellum in motility. NYGB 0.25 % agar plates were inoculated with the wild-type strain and the *fliC* and *flgE* mutants. After incubation at 28 °C for 72 h, both mutants showed a considerable reduction in their motility compared with the wild-type (Fig. 1a). This was expected because the same phenotype has been reported for these kinds of mutations in other bacteria (Murray & Kazmierczak, 2006; Young *et al.*, 1999). The complemented mutant strains showed a partial restoration of their motility, confirming that no other genes had been affected in the mutants (Supplementary Fig. S2).

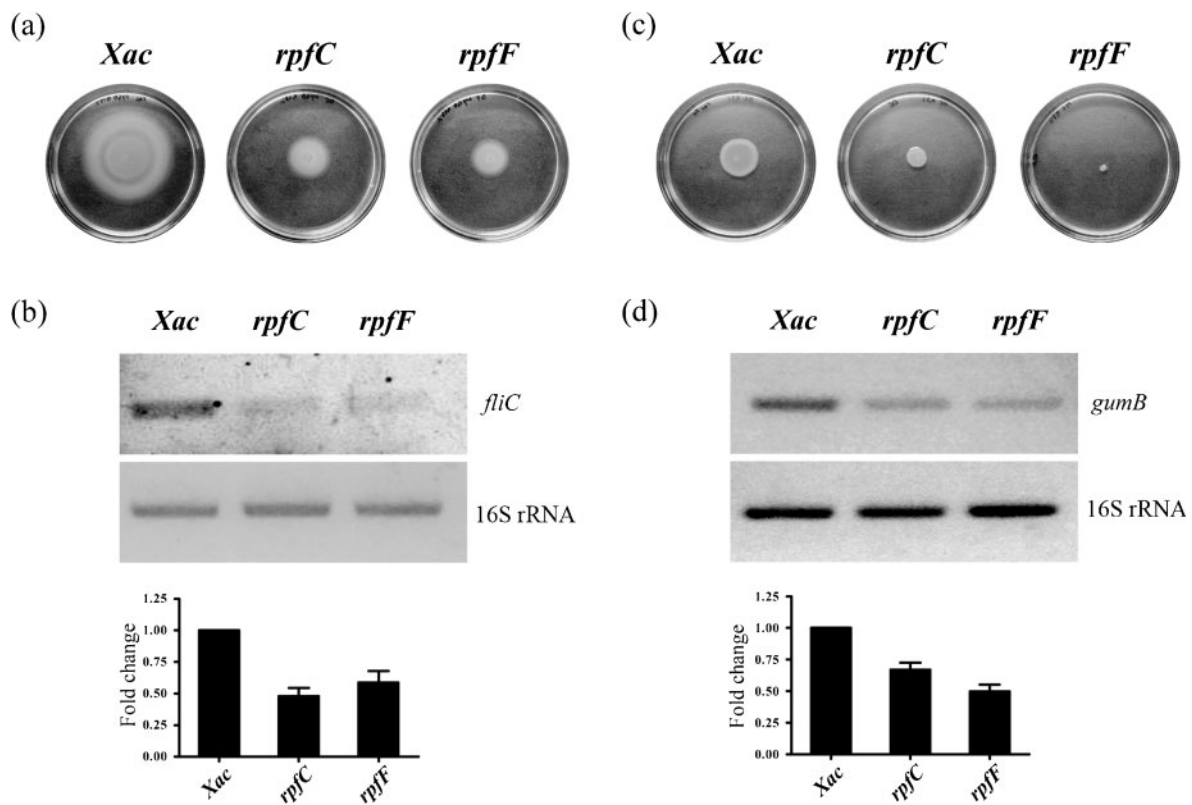


Fig. 2. Motility and gene expression in *Xac* strains with disruptions in the Rpf/DSF signalling system. (a) Flagellum-dependent swimming motility on 0.25 % agar NYGB plates. (b) Expression of the *fliC* gene assessed by semi-quantitative RT-PCR using cDNA from wild-type *Xac*, and *rpfC* and *rpfF* mutants grown in YMM medium to stationary phase ($n=3$). (c) Motility on 0.5 % PYM agar plates with 1 % glucose. (d) Expression of the *gumB* gene assessed by semi-quantitative RT-PCR in wild-type *Xac*, and *rpfC* and *rpfF* mutants grown in YMM medium to stationary phase ($n=3$).

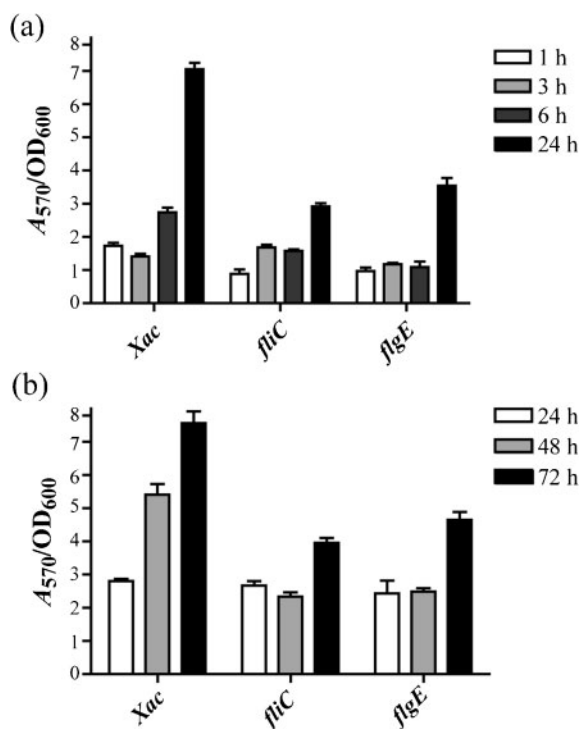


Fig. 3. Adhesion and biofilm development of *Xac* strains *in vitro*. (a) Adhesion in a 96-well polystyrene plate. Aliquots (150 μ l) of overnight cultures were used to inoculate the plates. OD₆₀₀ measurements and crystal violet (CV) assays were performed at 1, 3, 6 and 24 h after inoculation. (b) Biofilm development on wells of a 96-well polystyrene plate. Overnight cultures in nutrient medium were diluted in YMM to final OD₆₀₀ of 0.1. The OD₆₀₀ measurements and CV assays were performed at 24, 48 and 72 h after inoculation. Three independent experiments with six replicates for each strain were performed. Error bars represent SEM.

In *Xcc*, DSF signalling elevates the expression of a wide range of genes, including those involved in flagellum synthesis (He *et al.*, 2006). This prompted us to evaluate whether DSF signalling regulates swimming motility in *Xac*. The reduced motility of *rpfF* and *rpfC* mutants (Fig. 2a) and the reduced expression levels of *fliC* in both mutants (Fig. 2b) were indicative that flagellar-mediated swimming in *Xac* depends on cell–cell signalling. The *gumB* mutant showed a wild-type motility, indicating that xanthan has no role in *Xac* swimming motility (Fig. 1a). The *gumB* gene, as reported previously (Vojnov *et al.*, 2001), is also regulated by the DSF/Rpf system (Fig. 2d).

A second type of *Xac* motility is independent of the flagellum

The different *Xac* strains were also tested for motility on 0.5% agar PYM swarm plates. Both *fliC* and *flgE* mutants showed a comparable surface translocation to the wild-type (Fig. 1b) although the *gumB* (Fig. 1b) and *rpf* (Fig. 2c)

Fig. 4 (on facing page). Structural analysis of biofilms formed by *Xac* strains by CLSM. GFP-tagged cells were grown for 5 days in chambered cover slides and observed daily. (a) Panels (left to right) show the projection in the *x*–*y* plane at 3, 4 and 5 days p.i. for *Xac* wild-type, and *fliC* and *flgE* mutants. Magnification, $\times 1000$. Bars, 10 μ m. Arrows indicate some of the aggregates. (b) Mean thickness of the biofilm structure at different days after inoculation calculated by the COMSTAT program. Error bars represent SEM. (c) Biovolume of the biofilm structure at different days after inoculation calculated by the COMSTAT program. Error bars represent SEM. (d) Biofilm structures on day 5 after inoculation: projection in the *x*–*y* plane with a magnification of $\times 1000$ and a twofold digital amplification. (e) Biofilm structures at 5 days p.i.: projection in the *x*–*z* plane with a magnification of $\times 1000$ and a twofold digital amplification. Bars, 5 μ m. Arrows indicate groups of pioneer cells. Three independent experiments were carried out for each strain with essentially the same outcome.

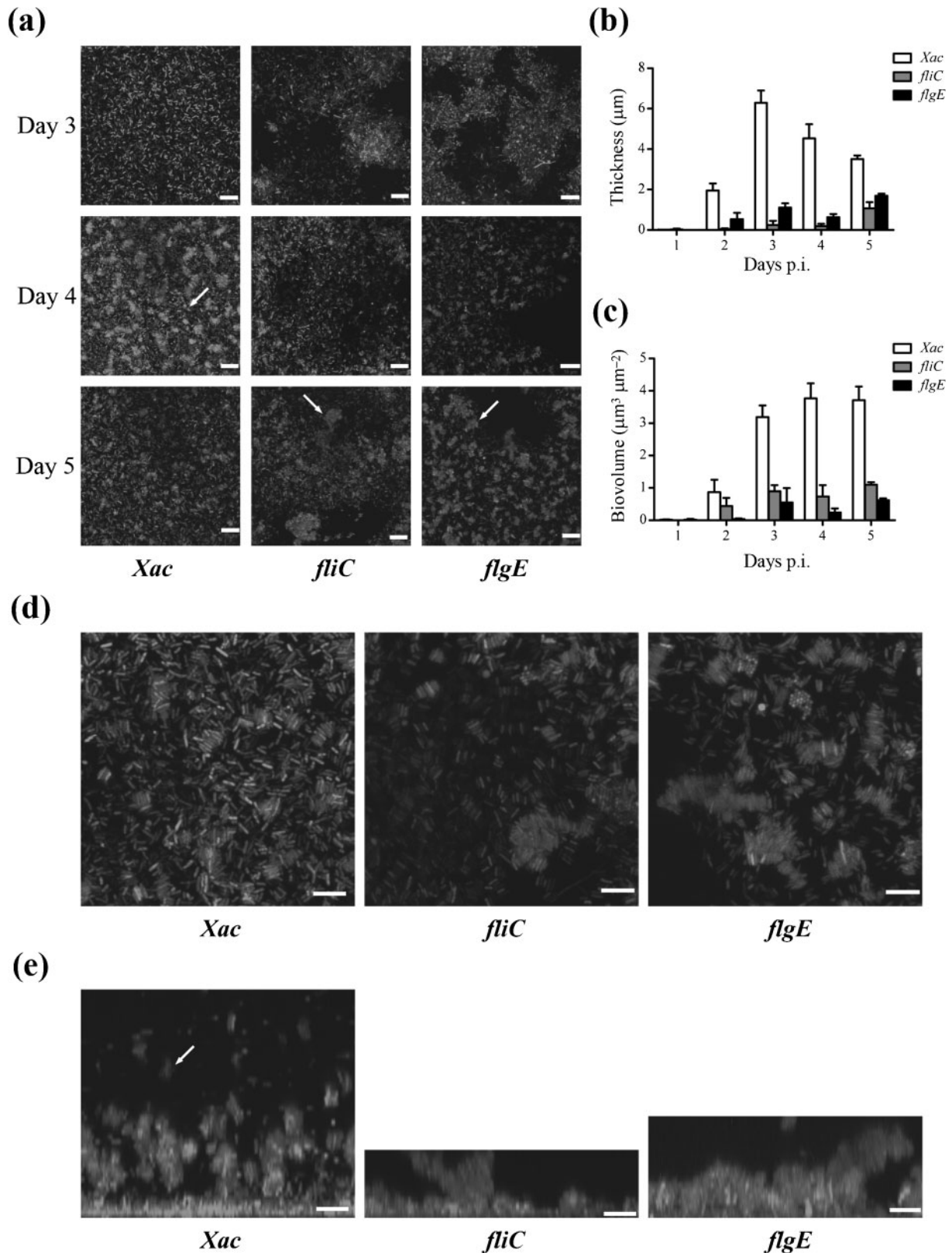
mutants showed a reduction in motility. Given these findings, under the conditions here, motility was not dependent on the presence of a flagellum, but was dependent on xanthan production and DSF signalling. Such motility has been termed ‘sliding’ (Horng *et al.*, 2002), and it has been proposed that EPS acts as a surfactant or surface-wetting agent to facilitate this type of movement (Murray & Kazmierczak, 2008).

Flagellar mutants show reduced attachment to abiotic surfaces and biofilm development

Adhesion to an abiotic surface and biofilm formation was assayed using 96-well polystyrene plates and CV staining. The *fliC* and *flgE* mutants showed reduced adherence throughout a 24 h experiment, suggesting that the intact flagellum is required for this stage (Fig. 3a). To follow biofilm development, a lower bacterial concentration (OD₆₀₀ 0.1) was used to inoculate the 96-well plates. Staining of the biofilm was performed at 24, 48 and 72 h. The wild-type *Xac* showed an increased biofilm biomass as revealed by CV staining while the biofilm biomass of the *fliC* and *flgE* mutants was reduced. However, these strains were still able to attach and develop a biofilm at 72 h (Fig. 3b).

Role of the flagellum in the structural development of *Xac* biofilms

We previously observed by CLSM that GFP-tagged *Xanthomonas* strains develop three-dimensional biofilm structures in static cultures in minimal medium, by using chambered cover slides (Rigano *et al.*, 2007b; Torres *et al.*, 2007). During the initial attachment *Xac* first contacted the glass surface in a polar fashion before becoming attached laterally. The colony of attached bacteria then spread over the entire well (Fig. 4a, day 3 and data not shown). After this first step, *Xac* developed microcolonies that increased in number (Fig. 4a). A more structured biofilm was formed from these microcolonies after 4 days (Fig. 4a). At higher



magnifications, it was possible to observe that the bacteria were linked predominantly through lateral interactions. After 5 days, wild-type *Xac* developed mushroom-type

structures separated by extensive water channels (Fig. 4e). At this time, some groups of cells (pioneer cells) spread from the biofilm to other locations (Fig. 4e).

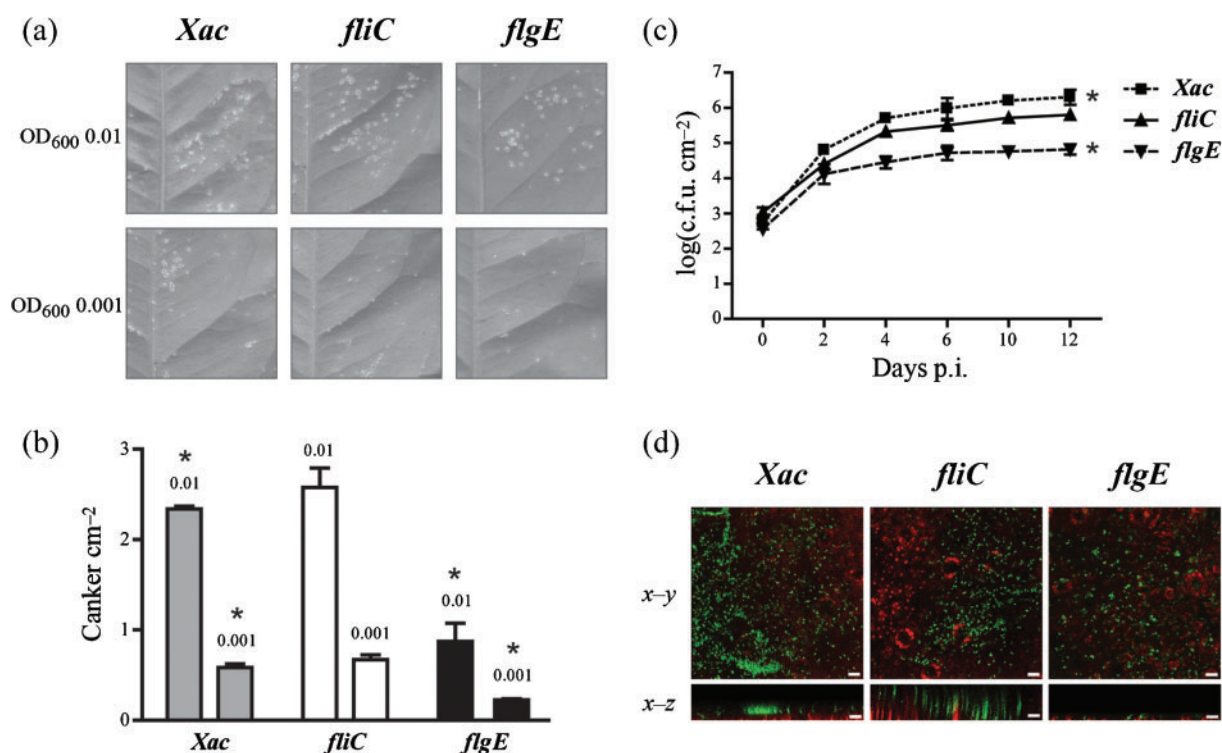


Fig. 5. Canker development, bacterial growth and biofilm formation on the surface of leaves infected with *Xac* strains by the spray method. (a) Lemon leaves at 22 days p.i. with 1×10^5 or 1×10^4 c.f.u. ml⁻¹. (b) Quantification of canker lesions in lemon leaves at 22 days p.i. Twenty leaves were assayed and the results are the means of four independent experiments \pm SD. The OD₆₀₀ of the suspensions used for inoculation is indicated above the bars. Asterisks indicate a statistically significant difference between the *flgE* mutant and the wild-type at both concentrations of bacterial suspension. (c) Growth *in vivo* of *Xac* strains on *C. limon* leaves infected with 1×10^6 c.f.u. ml⁻¹. Values are means of three independent experiments \pm SD. Datasets marked with an asterisk are significantly different according to the two-tailed Student's *t*-test ($P < 0.05$). (d) Biofilm structure analysis at 6 days p.i. using CLSM and GFP-labelled cells at a concentration of 1×10^6 c.f.u. ml⁻¹. The panels show projections in the *x*-*y* and *x*-*z* planes with a magnification of $\times 100$. Red indicates chlorophyll autofluorescence. Three independent experiments were assayed with essentially the same outcome.

Although the *fliC* and *flgE* mutants showed no apparent alteration in the first steps of cell attachment, they were not able to occupy the whole surface area of the chamber, in contrast to the wild-type strain (Fig. 4a, d). After 3 days, both mutants were able to develop aggregates (Fig. 4a, day 4) that later formed structures similar to those of the wild-type but thinner (Fig. 4d). Furthermore, these mutants failed to form a mature biofilm (Fig. 4e). These visual observations were confirmed by quantification of two variables, mean thickness and biofilm volume, by using the COMSTAT program (Fig. 4b, c).

The flagellum is involved in mature biofilm formation on leaf surfaces and is required for bacterial growth and canker development

To investigate if the flagellar mutants were affected in virulence, canker development, bacterial growth and biofilm formation, we evaluated the mutant strains in the

lemon leaves infection model. The plants were spray-inoculated with *Xac* wild-type, *fliC* and *flgE* mutant strains. Fig. 5(a) shows canker symptoms on lemon leaves 22 days p.i. at a concentration of 1×10^4 and 1×10^5 c.f.u. ml⁻¹. Quantification of canker lesions in lemon leaves was done at 22 days p.i. The *flgE* mutant was severely compromised in its ability to cause canker disease in citrus leaves (Fig. 5b). Comparison of bacterial numbers *in planta* revealed no significant statistical difference between *Xac* wild-type and the *fliC* mutant. However, the *flgE* mutant showed a statistically significant reduction in growth compared with the *Xac* wild-type (Fig. 5c).

When biofilm was analysed at 6 days p.i. on lemon leaves, the wild-type strain showed aggregation within a typical biofilm, as observed previously (Rigano *et al.*, 2007b) (Fig. 5d). However, biofilms from the *fliC* and *flgE* mutants developed in a more dispersed fashion with either few or no aggregated structures. The *flgE* mutant was more affected than the *fliC* mutant (Fig. 5d).

DISCUSSION

In the present study we have addressed the importance of motility and the role of the flagellum in the development of mature biofilm structures in *Xac*. Previous work from our group showed that a wild-type *Xac* strain was able to attach and develop a biofilm with an organized structure on both abiotic and biotic surfaces. Similar structures were also seen within the cankers of infected plants (Rigano *et al.*, 2007b). In addition, it has been shown that the xanthan polysaccharide in *Xac* is essential in the formation of microcolonies and the subsequent development of more complex structures (Rigano *et al.*, 2007b). In the present work, we show evidence that the flagellum is essential for the establishment of a mature biofilm in *Xac*, that EPS is required for a flagellum-independent type of motility ('sliding') and that both sliding motility and swimming motility (flagellum-dependent) are regulated by the *rpfII* DSF system.

In addition to swimming motility in low-percentage agar media, many bacteria exhibit swarming motility on plates with higher (0.5–0.6%, w/v) agar concentrations. Swarming motility is associated with changes in flagella number and/or placement, quorum sensing, and also with the production of wetting agents (Henrichsen, 1972; Kaiser, 2007; Overhage *et al.*, 2007). Both flagella and type IV pili contribute to swarming in *Pseudomonas aeruginosa* (Harshey, 1994; Rashid & Kornberg, 2000) and probably in other bacteria (Köhler *et al.*, 2000; Overhage *et al.*, 2007). In contrast, *Xac* is capable of flagellum-independent surface translocation on 0.5% agar PYM. We consider that this type of motility is more consistent with sliding rather than swarming. Sliding motility has been defined as 'a kind of translocation produced by expansive forces in a growing culture in combination with special surface properties of the cells resulting in reduced friction between cell and substrate' (Murray & Kazmierczak, 2008). Unlike swarming, sliding motility does not require flagella (Henrichsen, 1972). It is not known how DSF signalling and EPS impinge on the proposed sliding motility, although it is possible that EPS plays a role in modifying the properties of the agar surface to promote motility.

Xac does not exhibit twitching motility (unpublished observations), and consequently it is tempting to speculate that the type of motility described here as sliding has a role in the formation of initial microcolonies which occurs in a flagellum-independent manner. This remains an open question however, since the factors that we demonstrate to influence sliding are DSF signalling, which has a very pleiotropic effect in *Xanthomonas* spp., and the EPS xanthan, which probably also has a number of different roles in biofilm formation (Dow *et al.*, 2003).

Bacterial attachment, the formation of aggregates or microcolonies, and finally biofilm maturation have been recognized as the three stages of biofilm development that are responsive to diverse environmental signals (Stanley & Lazzerza, 2004). Mutations of the *fliC* or *flgE* gene

significantly reduced, but did not eliminate, initial attachment to both abiotic and biotic surfaces, suggesting that other genes may be involved in this process. CLSM revealed that in chambered slides, *fliC* and *flgE* mutants do not develop the structured organization seen in wild-type *Xac*. A typical biofilm contains bacteria tightly packed in hexagonal arrays separated by water-filled channels that allow nutrient flux between the towers of cells, and develops multicellular mushroom-shaped structures. The biofilms of the flagellar mutants lack water channels, which may explain why these mutants produce thinner structures that are reflected in the low CV staining. Overall, the data suggest that the flagellum plays a major role in the formation of the mature *Xac* biofilm. However, it remains unclear whether this deficiency is due to the absence of the flagellum structure or the absence of motility.

Similarly to the findings described here, a study in *Xcc* reported that the absence of flagellin affected the swimming motility of the bacteria as expected but did not alter virulence (Lee *et al.*, 2003). However, results obtained with other vascular pathogens such as *Ralstonia solanacearum*, *Pantoea stewartii* and *Dickeya dadantii* showed that the flagellum-dependent motility was required for biofilm formation and systemic colonization of the host plant (Herrera *et al.*, 2008; Jahn *et al.*, 2008; Tans-Kersten *et al.*, 2001). Although the mutation in the *fliC* gene in *Xac* affects the formation of biofilm structures, these strains are not significantly less virulent. The mutation in the *flgE* gene, in contrast, affects both the ability to form biofilms and virulence. As far as we are aware, this is the first study to establish the participation of FlgE in biofilm formation and virulence in a *Xanthomonas* species.

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