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Identification and characterization of biofilm formation-defective mutants of *Xanthomonas citri* subsp. *citri*

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Xanthomonas citri subsp. *citri* (*Xcc*) develops a biofilm structure both *in vitro* and *in vivo*. Despite all the progress achieved by studies regarding biofilm formation, many of its mechanisms remain poorly understood. This work focuses on the identification of new genes involved in biofilm formation and how they are related to motility, virulence and chemotaxis in *Xcc*. A Tn*5* library of approximately 6000 *Xcc* (strain 306) mutants was generated and screened to search for biofilm formation defective strains. We identified 23 genes not previously associated with biofilm formation. The analysis of the 23 mutants not only revealed the involvement of new genes in biofilm formation, but also reinforced the importance of exopolysaccharide production, motility and cell surface structures in this process. This collection of biofilm-defective mutants underscores the multifactorial genetic programme underlying the establishment of biofilm in *Xcc*.

INTRODUCTION

The bacterial phytopathogen *Xanthomonas citri* subsp. *citri* (formerly *Xanthomonas axonopodis* pv. *citri*) (*Xcc*) is the causal agent responsible for canker disease in citrus plants. Canker symptoms in leaves and fruits are characterized by surface-penetrating necrotic lesions surrounded by oily, water-soaked margins and yellow chlorotic rings (Brunings & Gabriel, 2003). This disease has a worldwide distribution and is hard to eradicate.

Previous studies have revealed that *Xcc* forms biofilms on both abiotic and biotic surfaces (Malamud *et al.*, 2011; Rigano *et al.*, 2007). Bacterial biofilm development is a temporal process involving transition through different stages of multicellular organization (Monds & O'Toole, 2009). The process starts with planktonic cells reaching and binding to a surface (Stoodley *et al.*, 2002). Later, attached cells form clusters or microcolonies through clonal growing and recruitment of other individuals. The microcolonies become larger and, as a result, macrocolonies appear. Inside these structures, bacteria are generally grouped as towers. They are held together by the presence of an exopolysaccharide (EPS) matrix that usually also contains dead cell debris, extracellular DNA and proteins. The last step of this process is dispersion, which happens when bacteria inside the macrocolonies are released (Tolker-Nielsen *et al.*, 2000).

Biofilm formation is associated with virulence in different bacterial pathogens. Over the past ten years the biofilm of *Xanthomonas* has been studied to comprehend the mechanisms underpinning this process in this group of bacteria. Some features of biofilm, known to be required in other micro-organisms, were also found necessary for the biofilm establishment of *Xcc*. Xanthan production (Rigano *et al.*, 2007), quorum sensing (Siciliano *et al.*, 2006), a filamentous haemagglutinin-like adhesin (Gottig *et al.*, 2009), flagellum synthesis (Malamud *et al.*, 2011), a UTP-glucose-1-phosphate uridylyltransferase (Guo *et al.*, 2010), LPS biosynthesis (Li & Wang, 2011a; Yan *et al.*, 2012) and

Abbreviations: CV, crystal violet; EPS, exopolysaccharide; T2SS, type II secretion system; T4SS, type IV secretion system; *Xcc*, *Xanthomonas citri* sp. *citri*.

Supplementary material is available with the online version of this paper.

glucan biosynthesis (Malamud *et al.*, 2012), a two-component signal transduction system encoded by *colS/colR* (Yan & Wang, 2011) and a LOV protein (Kraiselburd *et al.*, 2012) are the elements found to be a requirement for *Xcc* biofilm.

The focus of this study was to uncover essential components of the biofilm formation system.

By generating an EZ-Tn*5* library, approximately 6000 *Xcc* (strain 306) mutants were constructed and subsequently screened in order to find those impaired in biofilm formation. This screening enabled us to look for genes whose loss of function caused alterations in bacterial attachment and biofilm formation. A total of 28 genes were identified, including 23 novel genes and five genes that had already been associated with the process under study. We also explored the correlation between different types of virulence factors necessary for bacteria to develop the disease [motility, type II secretion system (T2SS) and chemotaxis], as well as assessing virulence of mutants in lemon leaves.

METHODS

Bacterial strains and culture conditions. WT *Xcc* strain 306 and mutant strains were grown at 28 °C in PYM medium or in a minimal medium (YMM) as described by Malamud *et al.* (2011). *Escherichia coli* strains were cultured at 37 °C in Luria–Bertani medium. When required, the antibiotics ampicillin and kanamycin where added to the growth media at a final concentration of 100 and 50 μg ml^{-1} respectively.

Mutant generation and screening for biofilm-defective strains. A transposon mutant library was generated with the EZ-Tn*5* <KAN-2> Transposon kit (Epicentre) following the manufacturer's instructions. A total of 6000 independent mutants were used in the biofilm-defective screening. Mutants were stored at −80 °C until use. The crystal violet (CV) technique was used to analyse biofilm development of the different strains on an abiotic surface. Briefly, bacterial strains were grown overnight in PYM nutrient medium and then inoculated into YMM. Aliquots of 150 μl were used to fill different wells of a microtitre plate and then incubated at 28 °C as previously described (Malamud *et al.*, 2011); the assay was performed in triplicate. These first candidates were subsequently subjected to the same assay three more times independently with eight replicates each time. Bacterial growth was measured in all cases before performing the CV staining. Selected biofilm-defective mutants were used for further characterization.

The assay was performed by growing cells at 28 °C for 24 h without agitation in YMM minimal medium as previously described (Malamud *et al.*, 2011; Rigano *et al.*, 2007).

Southern blot analysis. Southern blot was performed to determine the number of transposon copies in each mutant. Genomic DNA was extracted according to the manufacturer's instructions (Wizard Genomic DNA Purification kit; Promega), digested overnight with *Bam*HI (Promega) at 37 °C and subjected to electrophoresis on a 0.8 % agarose gel. The digested DNA was transferred to a Hybond-N+ membrane (Amersham Pharmacia Biotech). A PCR using the primers Tn5Foward (ACAGTAATACAAGGGGTGTTATGAGCCA) and Tn5Reverse (ACCAAACCGTTATTCATTCGTGATTG) was done to synthesize the probe. Probe labelling, hybridization and detection were performed using standard protocols.

Inverse PCR and identification of Tn*5* insertion sites. To identify the insertion site of the Tn*5* transposon, an inverse PCR was done. Briefly, total DNA from the selected mutants was digested with *Bam*HI (Promega) overnight at 37 °C. The enzyme was inactivated at 65 °C for 20 min. The DNA was incubated with T4 DNA ligase and the appropriate buffer in a final volume of 100 μl for 12 h at 16 °C. The products of the reactions were then used as a template in a PCR amplification using the primers Kan2 FP (ACCTACAACAAA-GCTCTCATCAACC) and Kan2 RP (GCAATGTAACATCAGA-GATTTTGAG). The PCR products were sequenced employing the same pair of primers and the resulting amplicon was subjected to BLAST analyses and alignment with *Xcc* 306 genomic sequence.

Plant growth conditions and inoculation. *Citrus limon* cultivar 'Eureka' was used as the host plant for *Xcc*. 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 and diluted in 10 mM $MgCl_2$ to a final concentration of 1×10^6 c.f.u. ml^{-1}. The symptoms were observed after 4 weeks of infection. The assay was repeated three times independently.

Cell motility assays. Swimming and sliding motility assays were carried out as previously described (Malamud *et al.*, 2011; Rashid & Kornberg, 2000). Briefly, bacteria were grown overnight in PYM medium and 3 μl of bacterial cultures with normalized OD_{600} were used to inoculate 0.25 % agar NYGB medium (0.5 % peptone extract, 0.3 % yeast extract and 20 ml glycerol l^{-1}) plates or 0.5 % agar PYM medium. Pictures of the motility plates were taken after 72 h of incubation at 28 °C.

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

Chemotaxis was tested as described by de Weert *et al.* (2002). Briefly, *Xcc* strains were grown in PYM medium and harvested in exponential phase. Cells were washed twice with YMM medium and resuspended in 12 ml 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). The resulting cell suspensions were poured into 60 mm diameter Petri dishes, creating a thin layer. Afterwards, 10 μl of grapefruit leaf extract was placed in the centre of each plate. After incubation from 30 min to 2 h at room temperature, the plates were analysed. The formation of a muddy zone around extracts was considered a positive chemotactic response (de Weert *et al.*, 2002).

RESULTS AND DISCUSSION

Screening for biofilm-defective mutants of *Xanthomonas citri* subsp. *citri*

To identify new genes involved in biofilm formation a mutant library was generated from *Xcc* strain 306. A total of 6000 EZ-Tn*5* mutants were screened using the CV technique and 96-well polystyrene microtitre plates as the abiotic surface to test adhesion ability. This method allowed us to screen a large number of mutants. In a first round, a total of 454 defective adhesion mutants were

found. Bacterial growth was verified before the CV staining since mutants' growth was fairly variable and this could affect the number of attached cells. Those mutants that showed growth impediment were rejected. Results were presented as a normalization of the value of biofilm formation against cell growth of all the mutants that we chose. Finally, 31 mutants that presented a significant reduction in adhesion but were not impaired in growth were identified ($P<0.0001$) (Fig. 1). YMM medium mimics the hostile environment of a leaf surface, where *Xcc* grows, almost lacking available nutrients.

Number of transposon copies, identification of the insertion site and relative position in the genome

Southern blots were performed to confirm the unique transposon insertions using the Tn*5* kanamycin resistance cassette as a probe against the genomic DNAs of the selected mutants. Almost all mutants had a single insertion of the transposon Tn*5* in their genome while only one of them had a double insertion (indicated as Tn*5* in Fig. S1, available in *Microbiology* Online). We did not detect any sign of the transposon in the WT strain genome (Fig. S1). The strain with two insertions was rejected for further analysis.

To determine the precise location of the insertion sites in the genome of these 30 mutants, an inverse PCR was carried out. The sequence analysis showed that in two cases (for genes *XAC2585* and *XAC3733*) the transposon was inserted in the same gene-encoding region, but in a different location. In both cases we chose one of the mutants for further work. Finally, a total of 28 Tn*5* biofilm-defective mutants were characterized. The relative positions in the genome of the Tn*5* insertion sites are shown in Fig. 2 and Fig. S2. Transposon insertion may cause a polar effect and this could affect the normal transcription of the genes around the Tn*5*. An analysis was performed for each mutant, taking into account the relative position of the transposon and the presence of promoter regions predicted by BProm (Softberry, http://linux1.softberry.com/berry.phtml) (Fig. S2 and Table 1). Ten of the total mutants showed a possible polar effect of the transposon insertion. In the rest of the mutants, the direction of the transposon is opposite to the direction of the downstream genes; as a result, it should not be polar mutation (Fig. S2 and Table 1). We decided to restore the gene function in two of the mutants to reject the possibility of the polar effect of the Tn*5* transposon. Complementations were designed considering each entire gene under the activity of its own promoter. The ability to attach to an abiotic surface was restored in both of the tested mutants: *XAC0618* and *XAC3733*. In the first one, the transposon did not produce a polar effect. In the second one, the putative polar effect was discarded (Fig. S3 and Table 1).

Characterization of the novel and known genes related to *Xcc* biofilm formation

Five of the 28 genes found in this screening were previously described to be involved during *Xcc* biofilm development (Table 1). These genes are involved in xanthan production (*gumB*, *galU* and *gumD*) (Guo *et al.*, 2010; Li & Wang, 2011b; Rigano *et al.*, 2007), in amino acid metabolism (*sahH*) (Li & Wang, 2011b) and in periplasmic glucan biosynthesis (*hrpM*) (Malamud *et al.*, 2012).

According to their ability to form biofilm we decided to group the 28 biofilm-defective mutants into three categories:

Group I, mutants mildly reduced: in this group the disrupted genes include *XAC0019* (outer-membrane protein), *XAC0966* (a gene involved in nucleotide metabolism), *XAC2301* (hypothetical protein) and *XAC3733* (a gene involved in transcriptional regulation).

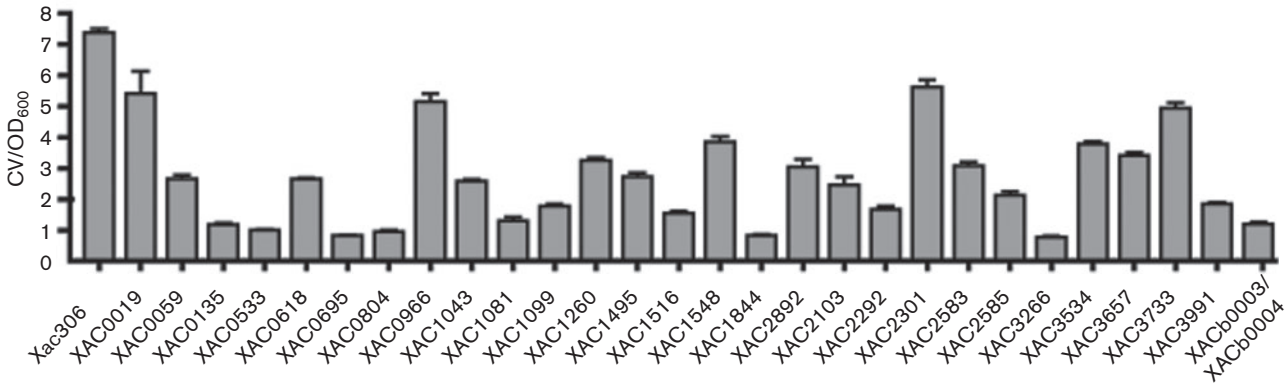

Fig. 1. Quantification of biofilm formation by CV technique. The EZ-Tn*5* mutants represented in the graphic are those with significant differences from the WT strain. The assay was performed in triplicate with eight replicates each time. Means and standard errors from one of three representative experiments with similar results are shown. Results are shown as the mean of the normalization of the value of biofilm formation against cell growth. Statistical significance was tested by Student's *t*-test. All strains showed significant differences from the WT strain, $P<0.0001$ %.

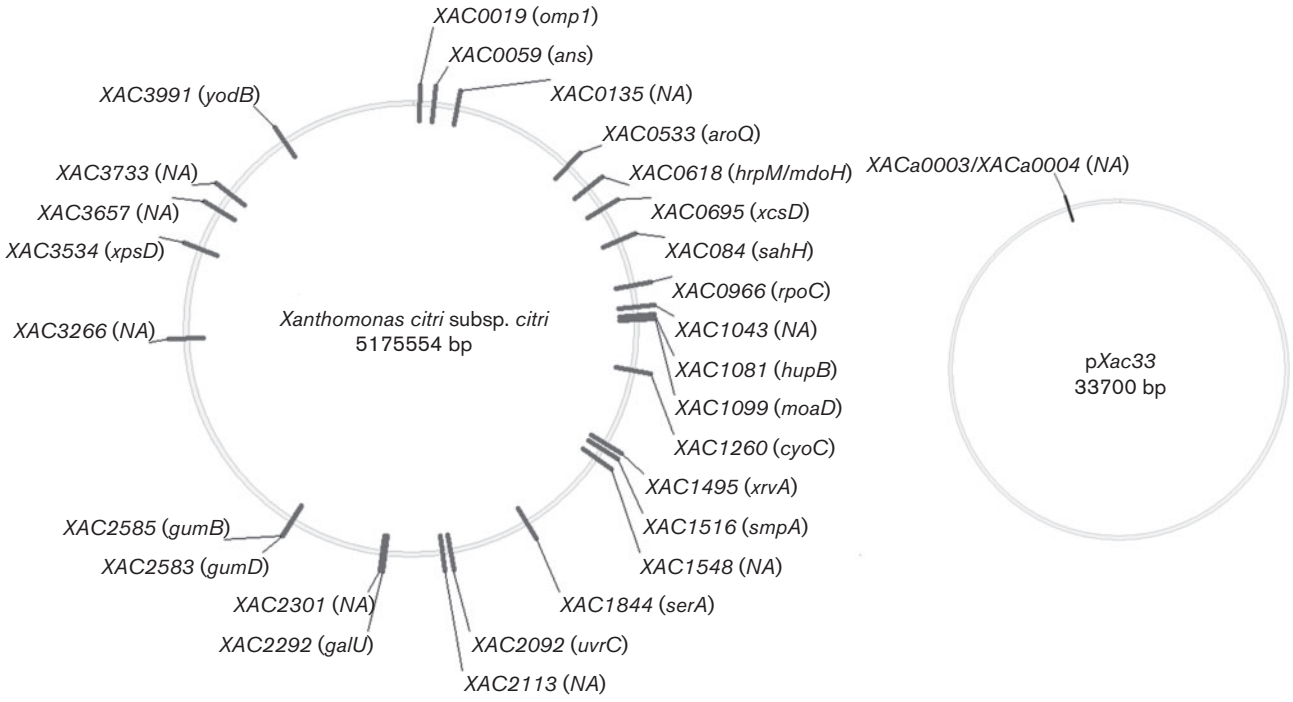

Fig. 2. Relative position of the biofilm-associated genes identified in this screening on the chromosome genome and on the pXAC64 plasmid of *Xanthomonas citri* subsp. *citri*.

Group II, mutants moderately reduced: *XAC0059* (a gene involved in amino acid metabolism), *XAC0618* (metabolic pathway), *XAC1043*, *XAC2113*, *XAC3657* (three hypothetical proteins), *XAC1260* (a gene involved in energy metabolism), *XAC1495* (histone-like protein), *XAC1548* (a gene involved in transcriptional regulation), *XAC2092* (a gene involved in nucleotide excision repair), *XAC2583* and *XAC2585* (two genes involved in xanthan production) and *XAC3534* (a gene encoding a structural protein of the T2SS).

Group III, mutants severely reduced: *XAC0135* (a sensor protein member of a previously un-described two-component system), *XAC0533* and *XAC0804* (genes involved in amino acid metabolism), *XAC0695* (a gene encoding a structural protein of the T2SS), *XAC1081* (histone-like protein), *XAC1098* (a gene involved in cofactor and vitamin metabolism), *XAC1516* (outer-membrane protein), *XAC1844* (a gene involved in amino acid metabolism), *XAC2292* (a gene involved in a metabolic pathway), *XAC3266* (hypothetical protein), *XAC3991* (a gene involved in energy metabolism) and *XACb0003*/*XACb0004* (an intergenic region in pXAC64 plasmid).

Biofilm-defective mutant affected in motility

Motility has been found to be important during biofilm formation in many bacteria. In previous work we observed that swimming flagellar-dependent motility in *Xcc* is necessary to develop a mature biofilm structure (Malamud *et al.*, 2011). We did not find any Tn*5* inserted in a flagellar gene. This fact may be related to the design of our screening. In previous work we demonstrated that the main difference between flagellar mutants and WT was observed after 3 days (Malamud *et al.*, 2011). Despite the fact that no flagellar-affected mutant was obtained in the screening, some of the mutants showed significant reduction in swimming motility (Table 1): *XAC0019*, *XAC0533*, *XAC0618*, *XAC0695*, *XAC0804*, *XAC1043*, *XAC1081*, *XAC1098*, *XAC1495*, *XAC2113*, *XAC2583* and *XAC3733*. Thus, these genes could be involved, direct or indirectly, in the flagellar functionality through biosynthesis, assembly of the flagella or energy production and supply for the flagella. *XAC2113* has a 54.4 % decrease in swimming motility compared to the WT strain. This gene encodes a 603 aa hypothetical protein with a predicted DUF3300 conserved domain (Pfam entry PF11737). This domain has a long hydrophobic segment, suggesting that *XAC2113* could be a membrane protein.

Another flagellum-independent type of locomotion described in *Xcc* is the sliding motility. The bacterium needs to produce EPS, which is supposed to play a role in modifying the properties of the agar surface to promote motility (Malamud *et al.*, 2011). A significant reduction in sliding motility was observed in some of the biofilm-defective mutants: *XAC0533*, *XAC1081*, *XAC1260*, *XAC2292*, *XAC2301*, *XAC2583*, *XAC2585* and *XAC3266*.

Table 1. *Xanthomonas citri* subsp. *citri* biofilm formation-defective mutants

Swimming and sliding assays: each test with three replicates was repeated four times with similar results. Means and standard errors of one of the assays from one representative experiment are shown.

Gene ID	Gene name	Insertion site (bp from ATG)	Possible polar effect	Definition	Swimming (%)	Sliding (%)	Virulence*	Chemotaxis (%)†	Categories
XAC0019	*omp1*	937	No	Outer-membrane protein	53.4±1.9	70.3±9	+	++	I
XAC0059	*asn*	757	No	Asparagine synthase-like protein	97.5±1	63.3±8	+++	++	II
XAC0135	NA	597/8	No	Two-component system sensor protein	71.5±1.9	75.4±14	+++	+++	III
XAC0533	*aroQ*	258/59	Yes	3-Dehydroquinate dehydratase	30.2±1.7	37.2±8.4	++	+++	III
XAC0618	*hrpM, mdoH*	446/7	No	Glucosyltransferase	25.5±1.8	98.9±3	−	+	II
XAC0695	*xcsD*	276	Yes	Type II secretion system protein D	54.9±0.9	60.7±6.3	+++	++++	III
XAC0804	*sahH*	381	No	*S*-Adenosyl-L-homocysteine hydrolase	31.7±3.2	77.7±15.5	+++	+++	III
XAC0966	*rpoC*	457	No	DNA-directed RNA polymerase subunit beta′	99.3±9.2	111±12.7	++	+++	I
XAC1043	NA	537	No	Hypothetical protein	57±1.3	65.4±2.4	+	++	II
XAC1081	*hupB*	141	Yes	Histone-like protein	17.3±2.8	5±0.5	−	−	III
XAC1098	*moaC*	160	Yes	Molybdopterin-converting factor chain 1	43.5±2.1	102.3±8.9	+++	+++	III
XAC1260	*cyoC*	249/250	No	Cytochrome *o* ubiquinol oxidase subunit III	73.4±3.8	13.1±3	+++	−	II
XAC1495	*xrvA*	219	No	Virulence regulator	47±1.3	94.6±4.5	+++	+	II
XAC1516	*smpA/omlA*	321	No	Outer-membrane protein	91.5±2.8	74.2±9.4	−	+++++	III
XAC1548	NA	141	Yes	GntR family transcriptional regulator	91.2±4.2	61.2±4.9	+++	+++++	II
XAC1844	*ser*	1063	No	D-3-Phosphoglycerate dehydrogenase	95.5±1.6	70.5±4	+++	+	III
XAC2092	*uvrC*	1836	Yes	Excinuclease ABC subunit C	79.6±2	100.9±9.7	++	++	II
XAC2113	NA	330	No	Hypothetical protein	54.4±1.1	69.3±10	+++	+++	II
XAC2292	*galU*	213	Yes	UTP-glucose-1-phosphate uridylyltransferase	57.5±4	14.13±6	+	−	III
XAC2301	NA	72	No	Hypothetical protein	88.6±0.3	33.5±1.3	+	+++	I
XAC2583	*gumD*	3	Yes	Undecaprenyl-phosphate glucose phosphotransferase	36.4±3.7	10.6±2.3	−	++++	II
XAC2585	*gumB*	420	Yes	Polysaccharide export outer-membrane protein	89±5.2	12.08±2.7	−	++++	II
XAC3266	NA	2252	No	Hypothetical protein	93±3.5	69.5±2.7	+	+++	III
XAC3534	*xpsD*	117	No	General secretion pathway protein D	97±1.3	102±4.8	+++	++	II
XAC3657	NA	201	No	Hypothetical protein	117.3±19.2	105±5.5	+++	+++	II
XAC3733	NA	482	Yes	NtrC family transcriptional regulator	31.72±4.4	85.6±15	+++	−	I
XAC3991	*yodB*	399/400	No	Cytochrome b561	81.5±6.2	86±2.4	+	++++	III
XACb0003/ XACb0004	NA	1512		Hypothetical protein/transposase	70.4±3.4	103±5.4	+++	++++	III

NA, not available.

*Virulence in lemon leaves, number of canker spots compared to WT: −, not detectable; +, barely detectable compared to the WT; ++, reduced compared to the WT; +++, normal.

†Induction of chemotaxis by grapefruit leaf extract, diameter and intensity: −, not detectable; +, barely detectable compared to the WT; ++, reduced compared to the WT; +++, slightly reduced compared to the WT; ++++, normal ; +++++, slightly increased compared to the WT.

The mutant *XAC2301* showed a reduction of 66.5 % in sliding motility compared to the WT strain. Although this hypothetical protein has no predicted domains, it is found in many species of the *Xanthomonas* genus. Mutants implicated in xanthan production, *XAC2292/galU*, *XAC2583/gumD* and *XAC2585/gumB*, also showed a reduction in sliding motility, confirming that xanthan is important for this type of movement. Surprisingly, *XAC2583/gumD* showed not only impairment in sliding motility, but also that its ability to swim was affected. In a previous biofilm screening (Li & Wang, 2011b), this mutant was also found to have shortcomings in both types of motilities, confirming our results. We observed that the production of EPS is affected in *XAC1260*, *XAC0533* and *XAC2301* (data not shown), although these genes are not known to be related with xanthan synthesis.

Another mutant, which showed less swimming and sliding, found in this screening has a mutation in the histone-like protein *hupB* (*XAC1081*). This is the first time that a histone-like protein, known as a global regulator of gene expression, has been related to biofilm development in *Xanthomonas*. This protein is highly conserved within the *Xanthomonas* genus and it belongs to a family of bacterial proteins (HU-IHF DNA-binding protein) implicated in the formation of a nucleoid-like structure that affects gene expression under certain conditions (Becker *et al.*, 2007). In addition, *XAC1495/xrvA* mutant was also affected in swimming, but not in sliding motility. This gene encodes a protein with an H-NS domain, also related to another kind of modular protein associated with the bacterial nucleoid. *XrvA* is conserved among most *Xanthomonas* species. In *Xanthomonas oryzae* pv. *oryzae*, it was described as a virulence regulator, a transcriptional repressor of genes involved in quorum sensing and EPS production (Feng *et al.*, 2009).

Biofilm-defective mutant affected in chemotaxis

Chemotaxis is the directed movement of cells in response to a chemoattractant gradient of chemical signals. The impact of chemotaxis in biofilm development depends on the bacterial species. Some works show a direct relationship between chemotaxis, attachment and biofilm development in *Agrobacterium tumefaciens* (Merritt *et al.*, 2007) and *Pseudomonas aeruginosa* (Schmidt *et al.*, 2011). However, in *E. coli*, chemotaxis was described as a non-critical process for a normal biofilm formation (Pratt & Kolter, 1998). So far, no relationship between chemotaxis and biofilm development has been demonstrated in *Xcc*. In a previous biofilm screening, four different genes involved in bacterial chemotaxis were found, but the authors did not characterize them (Li & Wang, 2011b). In order to establish a relationship between biofilm development and chemotaxis in *Xcc*, we performed a chemotaxis assay consisting of the measurement of bacteria migration ability towards grapefruit leaf extract. We found that, unlike WT strain, some biofilm-defective mutants were not able to move towards the attractant compound (Fig. 3, Table 1 and Fig. S4). There are two main causes of chemotaxis impairment: cells are non-motile or they have a mutation in some chemotaxis component.

Among the mutants affected in chemotaxis, seven of them were also affected in swimming motility: *XAC0019*, *XAC0618*, *XAC1043*, *XAC1081*, *XAC1495*, *XAC2292* and *XAC3733*. The other mutants that had shown problems in chemotaxis were able to swim: *XAC0059*, *XAC1260*, *XAC1844*, *XAC2092* and *XAC3534*.

xps and *xcs* T2SSs influence bacterial attachment and biofilm formation

Gram-negative bacteria are able to produce and secrete proteins implicated in virulence via the T2SS (Cianciotto, 2005). In *Xcc*, it has been demonstrated that extracellular enzymes contribute to canker symptom development by degrading the epidermal cell wall. Different kinds of protein are secreted via the T2SS, including protease, amylase and cellulase (Baptista *et al.*, 2010). Two mutants were found to contain the transposon insertion in the *XAC3534/xpsD* and in the *XAC0695/xcsD* genes. These genes are members of two different T2SSs, *xpsEFGHIJKLMND* and *xcsCDEFGHIJKLMND*, respectively. Both *xpsD* and *xcsD* mutant strains showed a lower amylase activity in comparison with WT strain (data not shown), confirming the hypothesis that both gene clusters encode functional components of T2SSs. This is the first time, to our knowledge, that a function has been assigned to the *xcsD* gene in *Xcc*.

In addition to bacterial adhesion to polystyrene microtitre plates analysed by crystal violet staining (Fig. 1), both *xcsD* and *xpsD* mutants were observed by confocal laser scanning microscopy (CLSM) using GFP-labelled bacteria, as described previously (see supplementary methods). After a 7 day time-course experiment on static cultures in minimal medium, the WT strain had developed mature biofilm structures with defined water channels. Both mutant strains (*xps*D and *xcsD*) were unable to reach the same type of structure as the WT strain (Fig. S5). The mutant

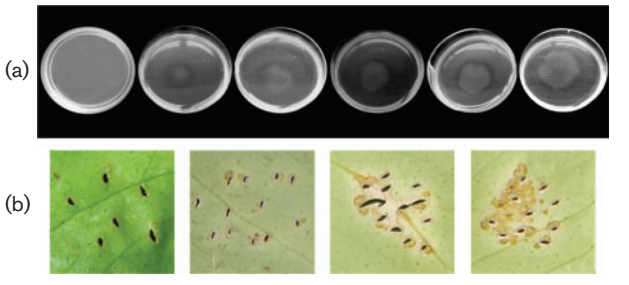

Fig. 3. Categories of mutants based on (a) chemotaxis ability, left to right from a non-detectable chemotaxis to a slightly increased chemotaxis compared to WT, and (b) symptoms produced in lemon leaves, left to right from non-detectable canker spot to a WT phenotype.

structures were more compact and showed no water channels (Fig. S5).

Virulence of the biofilm-defective mutants

To test if biofilm-defective mutants were able to cause similar symptoms to those caused by the WT strain, we inoculated young susceptible lemon leaves with all of them. Bacterial suspensions were swabbed on leaves (previously injured with a needle) to let bacteria enter into the mesophyll. Infected leaves were monitored up to 4 weeks or until the appearance of cankers (Fig. 3, Table 1 and Fig. S6).

Only five mutants did not cause any visible disease symptom: *XAC0618/hrpM*, *XAC1081/hupB*, *XAC1516/smpA*, *XAC2583/gumD* and *XAC2585/gumB*. *hrpM* and *gumB* have been previously demonstrated to be avirulent in young lemon leaves (Malamud *et al.*, 2012; Rigano *et al.*, 2007). *XAC1516/smpA* mutant was also found in a screening of genes involved in canker symptom development. The authors found that its absence led to a decrease of disease symptoms in grapefruit leaves (Yan & Wang, 2012). *smpA* encodes a lipoprotein located in bacterial outer membrane and it is possibly involved in maintaining the structural integrity of the cell envelope.

Five mutants revealed a significant reduction of symptoms produced in infected leaves: *XAC0019/omp1*, *XAC1043*, *XAC2301*, *XAC3266* and *XAC3991/yodB*. *XAC3266* encodes a hypothetical protein with no predicted conserved domains. Previous studies suggest that the protein encoded by *XAC3266* interacts with VirD4, the ATPase coupling protein of the type IV secretion system (T4SS) (Alegria *et al.*, 2005). A direct relationship between this system and biofilm development in *Xcc* has not been described yet. However, a former mutant screening also found a hypothetical protein related to biofilm formation that interacts with VirD4 (Li & Wang, 2011b). Both proteins share a conserved domain of approximately 120 amino acids that is present in all the VirD4-associated proteins (Alegria *et al.*, 2005). These results suggest that the T4SS in *Xcc* is associated with biofilm development.

XAC1043 and XAC2301 are both hypothetical proteins, highly conserved among *Xanthomonas* spp. The *XAC1043* gene encodes a 548 amino acid hypothetical protein that belongs to the peptidase_M48 family (Pfam entry PF01435). This type of proteins are Zn-dependent peptidases and were found to be associated with virulence *in vivo* and *in vitro* in *Porphyromonas gingivalis* (Walters *et al.*, 2009). XAC2301 does not have annotated conserved domains in any protein database.

Mutants found to be affected only in biofilm

Some of the biofilm-defective mutants found in this study did not show deficiencies with respect to other aspects under investigation (motility, virulence, T2SS functionality and chemotaxis). These mutants are *XAC0059/asn*, *XAC1548*, *XAC3657* and the intergenic region in pXAC64. According to KEGG (Kyoto Encyclopedia of Genes and Genomes, http://www.kegg.jp/), the asparagine synthase-like protein XAC0059/Asn takes part in five different pathways including 'microbial metabolism in diverse environments'. Since bacterial metabolism varies according to environmental conditions and bacterial lifestyle, the expression and function of this gene could change consistently with this fact. XAC1548 has been annotated as a GntR family transcriptional regulator, a DNA-binding protein responsible for the regulation of several biological processes. Ballering *et al.* (2009) described that the *Enterococcus faecalis* GntR protein was necessary during biofilm development. A member of the HutC/FarR subfamily (GntR family) was proposed for the first time to be linked with biofilm formation. They also showed that the null GntR mutant presented a 50-fold-reduced biofilm formation when compared to the WT strain. Furthermore, this gene was differentially expressed according to bacterial lifestyle; its expression increased when bacteria were part of biofilm structures. According to our results, *XAC1548* was still virulent when inoculated in lemon young leaves, contrasting with the observation made in grapefruit (*Citrus paradisi*). In this plant, the mutant was unable to produce symptoms (Yan & Wang, 2012). These data support the fact that the function of this transcriptional regulator varies according to environmental conditions. XAC3657 is a putative secreted protein conserved in *Xanthomonas* with no function assigned. To our knowledge, this is the first time that the *XAC3657* gene has been associated with biofilm development.

CONCLUSIONS

In this work, an EZ-Tn*5* mutant library with approximately 6000 *Xcc* (strain 306) mutants was constructed and used to look for new genes involved in biofilm development. We identified a total of 28 biofilm-defective mutants with single insertions and validated them by Southern blot to determine the EZ-Tn*5* insertion copy number. To address a possible polar effect of the transposon, *in silico* analysis of the downstream gene(s) and transposon orientation in each mutant was done. As well as the *in silico* analysis, two of the mutants were complemented and the ability to form biofilm was restored. We have already reported that *hrpM* complementation recovered glucan biosynthesis, swimming motility and virulence on citrus plants (Malamud *et al.*, 2012).

Genes that we found to be related to biofilm development are involved in diverse activities: xanthan production, amino acid synthesis, energy metabolism, DNA replication, transcription, membrane transport and signal transduction.

Five of the 28 identified genes are already described to be important on biofilm formation (*gumB*, *gumD*, *galU*, *hrpM*

and *sahH*). Three of them are defective in xanthan production (*gumB*, *gumD* and *galU*), reinforcing the importance of the EPS in biofilm formation (Rigano *et al.*, 2007; Dunger *et al.*, 2007; Guo *et al.*, 2010). These facts contribute to validating the approach used here. On the other hand, the 23 novel genes that we determined to be related to biofilm provide new insights into the genetic basis of this process in *Xcc*.

We examined all the mutants regarding three aspects that were already related to biofilm development: motility, chemotaxis and virulence. According to our results most of the mutants were affected in some of these issues, except four of them. This fact evidences the involvement of other phenotypic characteristics that affect this process.

In this work, chemotaxis is described for the first time in *Xcc*, showing that the bacterium is able to move towards an attractant component (grapefruit leaf extract). Furthermore, our data indicate that flagellum-mediated chemotaxis contributes to forming a mature biofilm.

Finally, two high-throughput screenings looking for virulence-deficient mutants in grapefruit and for novel genes involved in biofilm formation have been performed (Li & Wang, 2011b; Yan & Wang, 2012). Only two genes (*XAC0804* and *XAC2583*) were found in both biofilm screenings. This could be explained by considering that the conditions used to grow and to select the mutants were different. Some of the genes detected in our screening were also obtained in the one that aimed to find genes implicated in citrus canker symptom development: *XAC3534*/*xpsD*; *XAC1099*/*moaD*; *XAC0618*/*hrpM*; *XAC1548*/*gntR*; *XAC0019*/*omP1* and *XAC2292*/*galU*. The fact that we have found coincident genes between the two approaches shows the close relationship between the two processes: the importance of biofilm formation in the pathogenicity of *Xcc*.

Our finding, through wide-screening analysis, of global biofilm formation-related genes will provide a better understanding of this process and the disease development. Furthermore, it will provide tools for the development of crop-protection methods based on the interruption of key processes in *Xcc* pathogenesis.

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