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HrpM is involved in glucan biosynthesis, biofilm formation and pathogenicity in *Xanthomonas citri* ssp. *citri*

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SUMMARY

Xanthomonas citri ssp. *citri* (*Xcc*) is the causal agent of citrus canker. This bacterium develops a characteristic biofilm on both biotic and abiotic surfaces. A biofilm-deficient mutant was identified in a screening of a transposon mutagenesis library of the *Xcc* 306 strain constructed using the commercial Tn*5* transposon EZ-Tn*5* <KAN-2> Tnp Transposome (Epicentre). Sequence analysis of a mutant obtained in the screening revealed that a single copy of the EZ-Tn*5* was inserted at position 446 of *hrpM*, a gene encoding a putative enzyme involved in glucan synthesis. We demonstrate for the first time that the product encoded by the *hrpM* gene is involved in β-1,2-glucan synthesis in *Xcc*. A mutation in *hrpM* resulted in no disease symptoms after 4 weeks of inoculation in lemon and grapefruit plants. The mutant also showed reduced ability to swim in soft agar and decreased resistance to H_2O_2 in comparison with the wild-type strain. All defective phenotypes were restored to wild-type levels by complementation with the plasmid pBBR1-MCS containing an intact copy of the *hrpM* gene and its promoter. These results indicate that the *hrpM* gene contributes to *Xcc* growth and adaptation in its host plant.

INTRODUCTION

Xanthomonas citri ssp. *citri* (*Xcc*) is the causal agent of the destructive citrus canker disease, responsible for significant economic losses. *Xcc* enters the host plant leaves through the stomata or plant tissue lesions (Brunings and Gabriel, 2003). Canker symptoms on leaves and fruits are characterized by surface-penetrating necrotic lesions surrounded by oily, water-soaked margins and yellow chlorotic rings (Brunings and Gabriel, 2003). The canker itself, together with surviving populations of bacteria, constitutes the principal source of disease spread (Cubero and Graham, 2004).

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Bacteria live in communities, forming biofilm structures, where they are protected from adverse conditions (Davey and O'Toole, 2000). The cells are embedded in an exopolysaccharide (EPS) matrix that protects the organism and is essential for the disease cycle of pathogenic bacteria (Branda *et al*., 2005; Southey-Pillig *et al*., 2005). We have demonstrated previously the importance of EPS for *in vitro* and *in vivo* biofilm formation in *Xcc*, and its correlation with epiphytic fitness and canker development, by generating an *Xcc gum* mutant strain defective in xanthan EPS synthesis (Rigano *et al*., 2007b). In addition, flagella and flagellum-dependent motility have been shown to be important for initial adherence to surfaces, the development of a mature and structured biofilm and biofilm dispersion (Malamud *et al*., 2011). We have also demonstrated that diffusible signal factor (DSF)/*rpf* for regulation of pathogenicity factors cell-to-cell signalling in *Xcc* regulates the production of EPS, glucan and extracellular enzymes (Siciliano *et al*., 2006), as well as the synthesis of flagella and the dynamics of biofilm formation (Malamud *et al*., 2011).

The periplasmic space of Proteobacteria contains oligosaccharides that can be linear, branched or cyclic, and are important in the maintenance of cell equilibrium and osmotic adaptation (Miller *et al*., 1986). Glucans are a type of oligosaccharide that are synthesized when the environment is hypo-osmotic, preventing water from entering the cell (Miller *et al*., 1986). In addition, glucans play an important role in the interaction between bacteria and plants, both in symbiotic interactions in the cases of *Bradyrhizobium japonicum* (Bhagwat *et al*., 1999) and *Sinorhizobium meliloti* (Geremia *et al*., 1987) and in pathogenic interactions with *Agrobacterium tumefaciens* (Iñón de Iannino *et al*., 1998) and *Pseudomonas syringae* (Loubens *et al*., 1993). Although previous work has demonstrated that the nodule development B (*ndvB*) gene of *X. campestris* pv. *campestris* (*X. campestris*) is implicated in the synthesis of the cyclic glucans (Rigano *et al*., 2007a), the genes responsible for their synthesis in *Xcc* have not been identified to date. The study of the cyclic glucans of *X. campestris* has revealed new roles for these molecules in disease through the suppression of plant immune responses, as they have

been shown to induce susceptibility in *Nicotiana benthamiana* and *Arabidopsis thaliana* by suppressing callose deposition and delaying pathogenesis-related 1 (PR1) defence gene expression. These effects of cyclic glucans occur both locally and in a systemic fashion (Rigano *et al*., 2007a).

Different studies have shown that *X. campestris*, *Xanthomonas campestris* pv. *vesicatoria* (*Xcv*) and *Xcc* produce cyclic glucans (Minsavage *et al*., 2004; Talaga *et al*., 1996; Vojnov *et al*., 2001). *Xcc* produces a neutral cyclic glucan containing 16 β-1,2-linked glucose residues (Talaga *et al*., 1996). Little is known about glucan biosynthesis in *Xanthomonas* spp. The genome sequences of *Xcc* indicate the presence of two putative genes involved in glucan biosynthesis: *ndvB* (XAC4195) and hypersensitive response and pathogenicity (*hrpM*) (XAC0618). The former is known to be implicated in glucan biosynthesis in *X. campestris* (Rigano *et al*., 2007a). Both NdvB and HrpM proteins show no similarity with the periplasmic glucan biosynthetic enzymes characterized previously in the Rhizobiaceae family (Ielpi *et al*., 1990). The *hrpM* locus was first described in *Pseudomonas syringae* pv. *syringae* (*P. syringae*) by Mukhopadhyay *et al*. (1988), who associated it with the development of a hypersensitive response on nonhost tobacco plants and with virulence in bean. Later, the relationship of the *hrpM* gene of *P. syringae* with the membrane-derived oligosaccharides (*mdoH*) gene of *Escherichia coli*, which encodes a glucosyl transferase involved in the synthesis of osmoregulated periplasmic glucans (OPGs), was demonstrated (Loubens *et al*., 1993). Mutation in the *hrpM* gene leads to reduced virulence in *Xcv* (Minsavage *et al*., 2004) and to defective biofilm formation in *P. syringae* (Peñaloza-Vázquez *et al*., 2010). Although *hrpM* has 38% identity with *mdoH*, which is involved in the production of OPGs, experiments to evaluate the contribution of *hrpM* to glucan biosynthesis have not been conducted to date.

In this study, we present evidence that the *hrpM* gene product of *Xcc* is involved in the synthesis of a cyclic glucan, and that its absence has a critical influence on biofilm formation, virulence, growth *in planta*, motility and H_2O_2 tolerance.

RESULTS

Identification of *hrpM* in a screen for novel mutants affected in biofilm development in *X. citri* ssp. *citri*

In order to identify new genes involved in biofilm formation in the *Xcc* 306 strain, an EZ-Tn*5* transposon mutagenesis library containing approximately 6000 transformants was constructed. A screening based on the crystal violet technique was used to search for mutants impaired in the adhesion to polystyrene 96-well microtitre plate surfaces. Mutants showing a decrease in adhesion compared with the wild-type strain were selected and the transposon flanking regions were identified by inverse polymerase chain reaction (PCR). Briefly, genomic DNA was digested and the fragments were autoligated, and this DNA was used as a template in a PCR in order to determine the Tn*5* site of insertion. One of the isolated mutants had the Tn*5* insertion in the putative *hrpM* gene (Fig. 1a). A single copy of the transposon in the chromosomal DNA of the mutant was confirmed by Southern blot analysis (Fig. 1b).

Xcc and the *hrpM* mutant exhibited the same growth curve in liquid culture (data not shown); thus, the mutant showed no loss of viability compared with the wild-type strain. The *hrpM* mutant displayed reduced ability to attach to an abiotic surface (50% less than the wild-type) (Fig. 1c). The role of the HrpM protein in the structural development of *Xcc* biofilms was studied in more detail by observing green fluorescent protein (GFP)-tagged *Xanthomonas* strains (wild-type, *hrpM* mutant and c-*hrpM*) in a confocal laser scanning microscope. Figure 1d shows that, in contrast with the wild-type strain, the *hrpM* mutant was unable to occupy the whole surface area of the chamber, demonstrating a defective biofilm phenotype. The defect in biofilm formation of the *hrpM* mutant strain was restored by complementation with an intact copy of the *hrpM* gene and its promoter region (c-*hrpM*) (Fig. 1c,d), but not by transformation with the empty vector pBBR1-MCS (data not shown).

The *hrpM* gene is implicated in glucan production

In order to evaluate the production of cyclic glucan in the *hrpM* mutant, all the strains were cultured overnight and glucans were extracted with trichloroacetic acid (TCA) as outlined in Materials and methods. The extracts containing cell-associated cyclic glucans were analysed by BioGel P4 chromatography. The elution profiles for the cell-associated material from the wild-type strain *Xcc* 306, the *hrpM* strain and the c-*hrpM* strain are shown in Fig. 2a. Fractions 33–40 were pooled and considered as the cyclic glucan, taking as a reference the previously characterized elution profile obtained by Vojnov *et al*. (2001). The identity of the material in these pooled fractions with cyclic glucan was confirmed by thin layer chromatography (TLC). The TLC profile is shown in Fig. 2b, where the identity of the spots corresponding to cyclic glucans was determined using a glucan standard (Vojnov *et al*., 2001). The cyclic glucan associated with cells was clearly detected in the wild-type strain, whereas no cyclic glucan was observed in the *hrpM* mutant, thus confirming that the insertion of Tn*5* in the *hrpM* gene impaired the production of cyclic glucan. The cyclic glucan deficiency was fully restored by complementation *in trans* of the *hrpM* strain with the *Xcc hrpM* gene (Fig. 2).

Virulence is affected in the *hrpM* mutant

We then determined the contribution of the *hrpM* gene to the pathogenicity of *Xcc* by performing infection assays in lemon (*Citrus limon* cv. Eureka) and grapefruit (*Citrus paradisi* cv. Macfadyen) leaves. A bacterial suspension was swabbed onto leaves

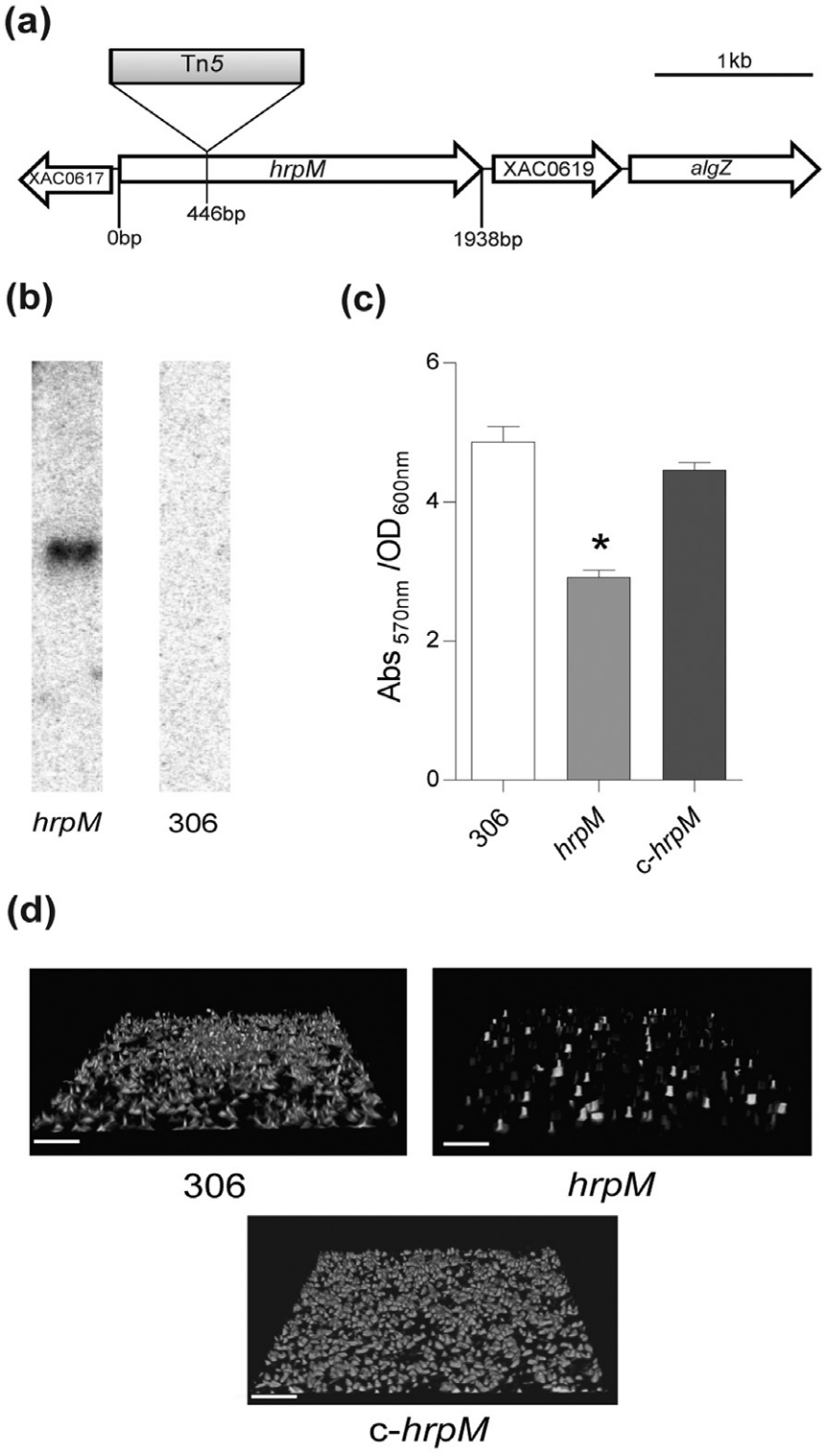

Fig. 1 The ability of the *Xanthomonas citri* ssp. *citri* (*Xcc*) *hrpM* mutant to develop biofilms is reduced. (a) The *hrpM* mutant was obtained by Tn*5* mutagenesis. The insertion of the Tn*5* transposon was located around the 446th nucleotide of the *hrpM* coding sequence and was determined by inverse polymerase chain reaction (PCR). (b) Southern blot hybridization of the *Xcc* and *hrpM* strains; genomic DNA was digested and hybridized with the probe. (c) Cell adhesion assay in 96-well polystyrene plates. Three independent experiments with six replicates for each strain were performed. Error bars represent the standard error of the mean (SEM); the data point marked with an asterisk is significantly different from the control *Xcc* 306 ($P < 0.001$). (d) Biofilm formed by *Xcc* 306, the *hrpM* mutant and the complemented strain c-*hrpM*. Green fluorescent protein (GFP)-tagged cells were grown in chambered coverslides for 7 days. Magnification, ×1000; scale bars, 20 μm.

previously injured with a needle to allow bacteria to enter the mesophyll. After 4 weeks, both kinds of citrus leaves developed disease symptoms when infected with the wild-type, but not with the mutant strain. Complementation with the *hrpM* gene fully restored the virulence in both plant species (Fig. 3a,b). We also performed an infection assay by pressure infiltration, and found that the *hrpM* mutant strain was unable to cause disease symptoms using this second method of inoculation (Fig. 3c).

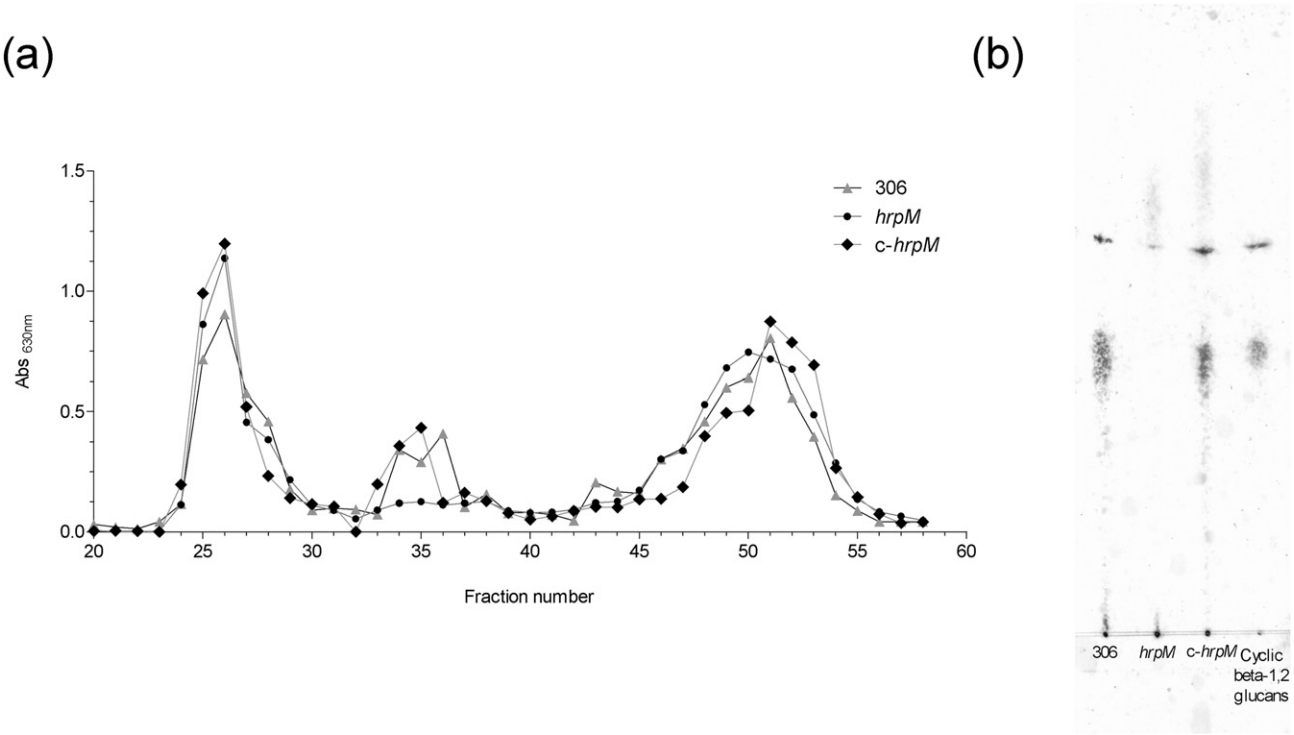

Fig. 2 Analysis of cyclic glucan production. (a) Size-exclusion chromatography on BioGel P4 column profiles for each strain of this study. (b) Thin layer chromatography (TLC) analysis of fractions 33–40 from *Xanthomonas citri* ssp. *citri* (*Xcc*) 306 (lane 1), *hrpM* (lane 2) and c-*hrpM* (lane 3), and purified cyclic β-1,2-glucan as a control (lane 4).

Growth of the three strains in lemon plants was compared. As shown in Fig. 3d, significant differences were observed between the *hrpM* mutant and the wild-type *Xcc* 306 strain. Seven days post-inoculation (dpi), the *hrpM* mutant was undetectable. At 13 dpi, the wild-type strain had grown by more than three orders of magnitude, whereas the *hrpM* mutant had not. The growth of the c-*hrpM* strain was similar to that of the wild-type strain. The difference observed between the two strains at 13 dpi is probably caused by the partial loss of the pBBR1-MCS::*hrpM* plasmid.

The *hrpM* mutant is impaired in hydrogen peroxide (H_2O_2) survival

One of the earliest plant responses after pathogen recognition is the production of reactive oxygen species (ROS), such as H_2O_2, which possesses antimicrobial activities. This first step of the plant response could be attributed to their ability to develop a biofilm. To measure the effect of *hrpM* disruption on the survival of the bacteria, wild-type and cyclic glucan-deficient strains were assayed. PYM-agar plates containing H_2O_2 were used to study the sensitivity to ROS. There was a significant difference in survival between the *Xcc* 306 and *hrpM* mutant strains (Fig. 4a,b,d). The growth inhibition area of the *hrpM* mutant was significantly larger than that of both the wild-type *Xcc* and c-*hrpM* (Fig. 4c,d). Thus, the reduction in resistance to oxidative stress of the *hrpM* mutant, one of the common stresses encountered by bacteria within their host plants, highlights the importance of periplasmic cyclic glucan during the initial steps of the infection process.

Flagellum-dependent motility is impaired in the *hrpM* mutant

As described by Malamud *et al*. (2011), bacterial motility is essential for biofilm formation and virulence. Both sliding and swimming motility were investigated in the *hrpM* mutant, which is affected in these processes. Sliding and swimming plates were inoculated with the different strains. After 72 h, no difference was detected with regard to sliding motility. This observation is related to the fact that the amount of EPS production did not vary significantly among the strains (data not shown). By contrast, the *hrpM* mutant was essentially nonmotile in the swimming plates (Fig. 5).

DISCUSSION

In order to look for new genes involved in biofilm development, we screened a Tn*5* transposon library for mutants affected in this trait. One of the isolated mutants has Tn*5* inserted in the *hrpM* gene, a gene putatively involved in glucan biosynthesis. Through TCA extraction, gel filtration and TLC analysis, we have demonstrated in this work that the *hrpM* gene product in *Xcc* is indeed

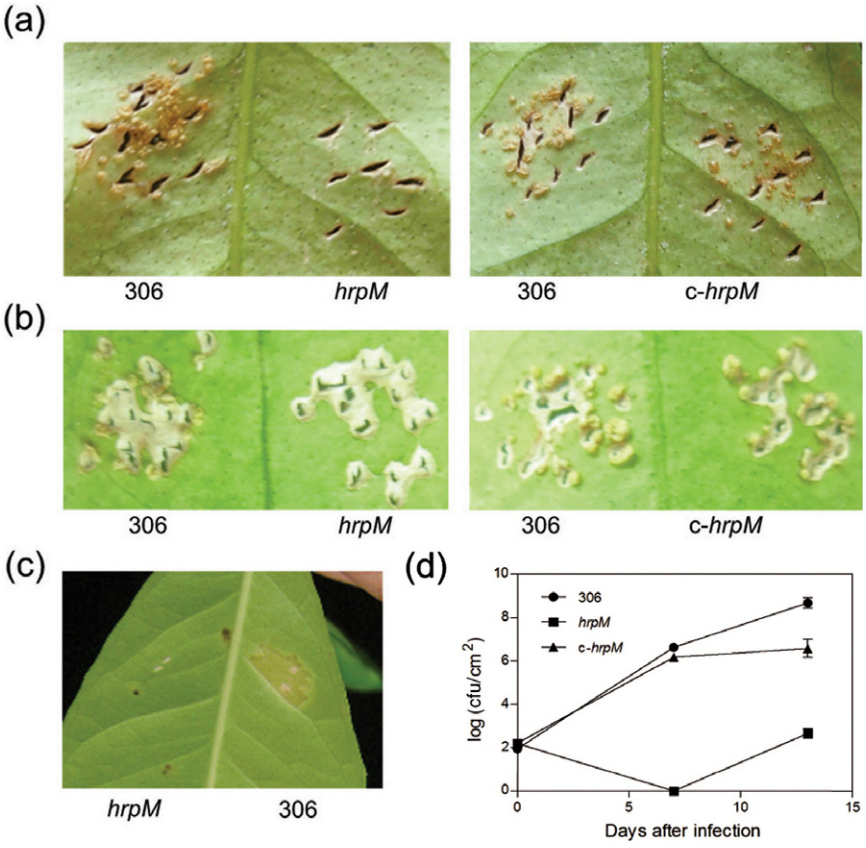

Fig. 3 Pathogenicity assays. (a) Bacterial suspensions of 1×10^6 colony-forming units (cfu)/mL of *Xanthomonas citri* ssp. *citri* (*Xcc*) 306, *hrpM* and c-*hrpM* were swabbed on the surface of injured lemon leaves (*Citrus limon* cv. Eureka); the photographs were taken 4 weeks after infection. (b) A similar assay was performed using *Xcc* 306, *hrpM* and c-*hrpM* on grapefruit leaves (*C. paradisi* cv. Macfadyen). (c) Bacterial suspensions of 1×10^6 cfu/mL of *Xcc* and *hrpM* were injected inside lemon leaves; photographs were taken 2 weeks after infection. (d) *In vivo* growth of *Xcc* strains on lemon leaves infected with 1×10^8 cfu/mL. Three independent experiments were carried out for each strain with essentially the same results.

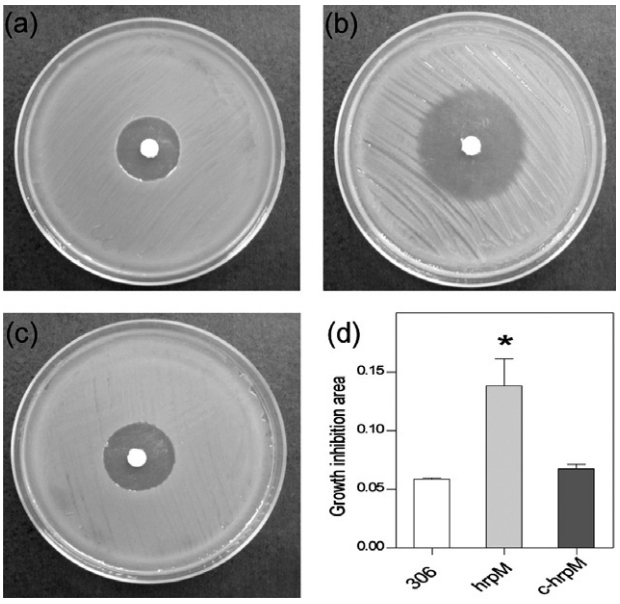

Fig. 4 *Xanthomonas citri* ssp. *citri* (*Xcc*) 306, *hrpM* and c-*hrpM* sensitivity to H_2O_2. After 48 h of incubation at 28°C, growth inhibition areas were determined. (a) Wild-type strain; (b) *hrpM*; (c) c-*hrpM*; (d) measurement of growth inhibition areas (values presented as the mean ± standard error, $n = 3$). Asterisk indicates a significant difference relative to the 306 control ($P < 0.005$).

involved in the production of the already reported cyclic β-1,2-glucan (Talaga *et al*., 1996). Previous studies in *P. syringae* have shown that glucans are associated with biofilm formation and the *hrpM* mutant is known to be impaired in this process (Peñaloza-Vázquez *et al*., 2010). However, to our knowledge, this is the first study to establish the participation of the *hrpM* gene product in glucan production beyond *in silico* studies.

Our results indicate that the *hrpM* gene product is involved in *Xcc* virulence. We showed that the *hrpM* mutant strain was unable to develop disease not only in lemon, but also in leaves of a more susceptible host, grapefruit. Depending on the method applied to infect plants, it is possible to determine an agent's contribution to different stages in the infection cycle. Swabbing in injured leaves mimics the *in vivo* entry of bacteria through natural openings, such as stomata, or through lesions. Pressure infiltration introduces bacteria mechanically inside the leaf increasing the efficiency in causing canker symptoms. The *hrpM* mutant showed no symptoms in either plant species, regardless of the infection method, suggesting that bacteria require the *hrpM* gene product not only inside, but also outside, leaf tissue. It is known that plant-associated biofilm formation is correlated with bacterial pathogenicity. Our results show that bacteria lacking glucans present lower adherence to abiotic surfaces, slower growth *in planta* and the inability to produce canker

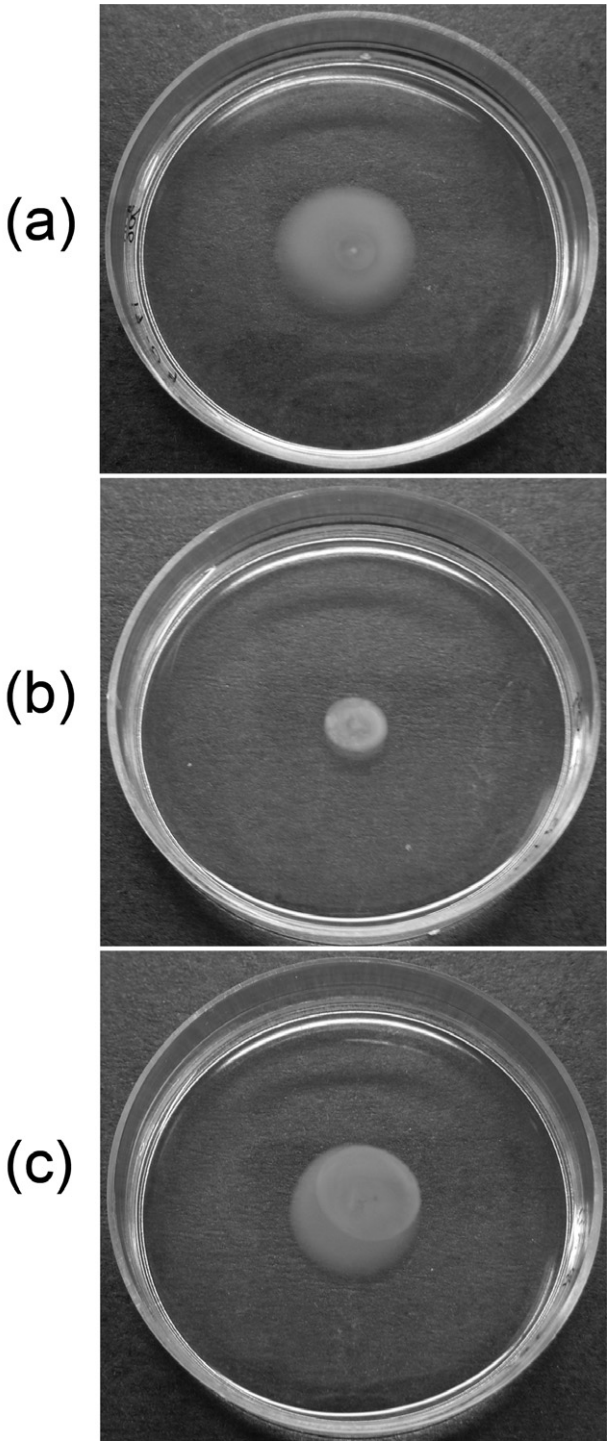

Fig. 5 Flagellum-dependent motility. Swimming motility of *Xanthomonas citri* ssp. *citri* (*Xcc*) (a), *hrpM* (b) and c-*hrpM* (c) on 0.25% (w/v) agar NYGB plates.

spots. As a consequence, it can be concluded that these compounds play an important role in biofilm formation and, hence, in bacterial survival on leaves for *Xcc*. Although bacteria inside leaves persisted for some time, the mutant deficient in glucan synthesis never reached wild-type strain levels, remaining at low concentrations.

Reactive oxygen species are a host defence mechanism to kill invading microbes in the early stages of infection. Here, we showed that the glucan *hrpM*-defective mutant was significantly more sensitive than the wild-type *Xcc* strain to H_2O_2, indicating that bacterial glucan is important for resistance to oxidative stress. Increased H_2O_2 sensitivity might explain the initial drop in bacterial numbers in *hrpM*-infected leaves, followed by a partial recovery by day 13. Nevertheless, the role of glucan in pathogen virulence should not rule out its possible influence on host defence. For example, glucan has been shown to mediate the suppression of plant defence reactions (Rigano *et al*., 2007a).

The deficiency in glucan production has been shown to cause pleiotropic phenotypes in other bacterial species, such as impaired antibiotic resistance and motility (Douglas *et al*., 1985; Geremia *et al*., 1987; Mah *et al*., 2003; Minsavage *et al*., 2004). Here, we showed that disruption of the *hrpM* gene provoked reduced motility in *Xcc*. This phenotype could be associated with the presumed role of cyclic glucans in plasma membrane stabilization and assistance in the correct assembly of membrane protein complexes from the periplasmic space (Briones *et al*., 2001; Peñaloza-Vázquez *et al*., 2010). Reduced swimming of *hrpM* mutants suggests a possible dysfunction in the flagellum, whose assembly is known to take place in the periplasmic space (Nambu and Kutsukake, 2000). Further experiments need to be carried out to analyse the integrity of flagella in order to understand how motility and pathogenicity are affected in the *hrpM* mutant strain.

Glucans are associated both within the cells (periplasmic cyclic glucans) (Talaga *et al*., 1996; Vojnov *et al*., 2001) and in culture supernatants (Amemura and Cabrera-Crespo, 1986; Vojnov *et al*., 2001; York, 1995). In other bacterial species, the mechanism of glucan export is well known. For example, in *Rhizobium meliloti*, the NdvA protein is responsible for this process (Stanfield *et al*., 1988). Nevertheless, the mechanism by which glucans are secreted from cells to the culture supernatant in *Xanthomonas* is still unknown.

In conclusion, this work provides new insights into the genetic determinants involved in biofilm formation and glucan biosynthesis. Our experiments associate these processes with tolerance to stress conditions, survival to H_2O_2 and virulence in *Xcc*. However, further studies should be conducted on the role of glucans in *Xcc* physiology, as well as on the individual contribution of each of the genes involved in cyclic glucan synthesis.

EXPERIMENTAL PROCEDURES

Bacterial strains and growth conditions

Xanthomonas strains were cultured at 28 °C with shaking at 220 rpm in peptone–yeast extract–malt extract (PYM) medium (Cadmus *et al*., 1976)

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

Strain or plasmid	Relevant properties		Source
E. coli DH5α	*hsdR, recA, lacZYA*, Φ80 *lacZ* ΔM15		GIBCO BRL
Xcc 306	Wild-type strain Amp^r		da Silva *et al*. (2002)
hrpM	Amp^r; Km^r		This work
c-hrpM	Amp^r; Km^r, Cm^r		This work
pRU1319	Gm^r		Allaway *et al*. (2001)
pBBR1-MCS	Cm^r		Kovach *et al*. (1994)
Primer name			
Tn*5* foward	ACAGTAATACAAGGGGTGTTATGAGCCA	Southern probe	This work
Tn*5* reverse	ACCAAACCGTTATTCATTCGTGATTG	Southern probe	This work
KAN-2 FP-1	ACCTACAACAAAGCTCTCATCAACC	Inverse PCR	EPICENTRE
KAN-2 RP-1	GCAATGTAACATCAGAGATTTTGAG	Inverse PCR	EPICENTRE
HrpM-compl foward	TTGAATTCCAACGCCAAAGCCCCGTCG	Complementation	This work
HrpM-compl reverse	TTGGATCCTCATCCAGCTCCGCTTGCG	Complementation	This work

Km^r, Amp^r, Gm^r, Cm^r indicate kanamycin, ampicillin, gentamycin and chloramphenicol resistance, respectively. PCR, polymerase chain reaction.

or Y minimal medium (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*., 2007b). *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 Scientific Corp., Madison, WI, USA) at 600 nm. When required, the antibiotics ampicillin (100 μg/mL), kanamycin (50 μg/mL), gentamycin (100 μg/mL) and chloramphenicol (35 μg/mL) were added to the growth media.

Transposon mutagenesis, transposon insertion mapping of *Xcc hrpM* and Southern blot

The mutant was generated using the EZ-Tn*5*™ <KAN-2> Tnp Transposome™ Kit (Epicentre, Madison, WI, USA). The transposon Tn*5* was inserted by electroporation according to the manufacturer's instructions. The colonies obtained were selected in PYM medium supplemented with kanamycin (50 μg/mL), and transferred individually to another plate. After 1 day at 28 °C, colonies were picked into wells of a 96-well microtitre plate containing PYM medium with kanamycin and 20% (v/v) glycerol. After the cells had grown, the plates were stored at −80 °C.

To identify the gene disrupted by the Tn*5* transposon, total genomic DNA was isolated using a Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA) according to the manufacturer's instructions, and digested with *Bam*HI. Fragments were autoligated and an inverse PCR was performed using Tn*5* forward and Tn*5* reverse primers (Table 1). The amplified fragment was sequenced and used in BLAST searches with NCBI/Blast.

A Southern blot was performed to verify the number of insertions of the transposon in the mutant genome. Briefly, *Xcc* and *hrpM* genomic DNAs were digested with *Bam*HI and subjected to electrophoresis on a 0.8% (w/v) agarose gel. The DNAs were transferred to Hybond-N^+ (Amersham, Little Chalfont, Buckinghamshire, UK) and hybridized with a ^{32}P-labelled probe, which was amplified by PCR, using the primers Tn*5* forward and Tn*5* reverse (Table 1) and genomic DNA from another Tn*5* mutant.

Complementation analysis of the *hrpM* mutant

To complement the *hrpM* mutant, a 2500-bp DNA fragment containing the entire open reading frame of XAC0618 and a 560-bp sequence upstream of the start codon was PCR amplified (Table 1). The PCR product was cloned into a pGEMT-easy vector (Promega) and digested with *Bam*HI and *Eco*RI. This fragment was ligated into partially digested pBBR1-MCS (Kovach *et al*., 1994). This construct was used to transform the mutant *Xcc* hrpM by electroporation, as described previously (Malamud *et al*., 2011).

Biofilm formation assay

The crystal violet technique was used to analyse the attachment of the different strains to an abiotic surface (O'Toole and Kolter, 1998). Briefly, bacterial strains were grown overnight in PYM nutrient medium, and inoculated into YMM to an optical density at 600 nm (OD_{600}) of 0.1. Aliquots of 150 μL were used to fill the different wells of a microtitre plate and then incubated at 28 °C for 24 h. To confirm similar bacterial growth, OD_{600} was measured before the adhesion assay was performed. The medium was gently removed using a pipette; the microtitre plate was washed with 0.9% NaCl, and stained using 0.1% (w/v) crystal violet solution. After 30 min of incubation, the unbound crystal violet was removed and the wells were washed twice with distilled water. The crystal violet of each well was dissolved by adding 150 μL of 70% (v/v) ethanol, and the absorbance was measured with a microplate reader at 560 nm.

In vitro analysis of biofilm formation by confocal laser scanning microscopy

All the strains used in this work were transformed by electroporation with pRU1319 (Allaway *et al*., 2001) carrying GFPuv. For *in vitro* experiments, these strains were grown at 28 °C on PYM supplemented with gentamycin. Aliquots of 500 μL were transferred to chambered coverglass slides containing a 1-mm-thick borosilicate glass (no. 155411; Lab-Tek, Nunc, Penfield, NY, USA), as described previously (Malamud *et al*., 2011). Cultures were diluted 1:1000 in YMM and grown in the chambers for 7 days at 28 °C. Biofilm formation was monitored with an Olympus Fluo View 1000 confocal laser scanning microscope (Center Valley, PA, USA). Three-dimensional images were generated with Image J 1.41 software from the National Institutes of Health (http://rsbweb.nih.gov/ij/download.html).

Swimming motility assay

Motility assays were carried out as described previously (Malamud *et al*., 2011; Rashid and Kornberg, 2000). Briefly, bacteria were grown overnight in PYM medium, and 3 μL of bacterial cultures with a normalized OD_{600} were used to inoculate 0.25% (w/v) agar NYGB medium [0.5% (w/v) peptone extract, 0.3% (w/v) yeast extract and 20 mL/L glycerol] plates. Photographs of the motility plates were taken after 72 h of incubation.

H_2O_2 assay

Bacteria were grown overnight in PYM medium, OD_{600} was measured and 100 μL of the cells were spread on PYM-agar plates with a swab. Whatman paper discs embedded with 3 μL of 100 mM H_2O_2 were placed over the PYM-agar plates. After 48 h of incubation at 28 °C, photographs of the plates were taken and the inhibition halo area was measured using ImageJ 1.41 software. Data were normalized to the total area of growth.

Plant growth conditions and inoculations

Citris limon cv. Eureka was used as the host plant for *Xcc*. Plants were kept in a room at a controlled temperature and a photoperiod of 16 h. *Xcc*, *hrpM* and c-*hrpM* were grown in PYM with the appropriate antibiotics and diluted in sterile distilled water to a final concentration of 1×10^6 colony-forming units (cfu)/mL. Bacterial suspensions were infiltrated into leaves and also swabbed onto injured leaves of lemon trees and grapefruit (*C. paradisi* cv. Macfadyen). The symptoms were observed after 2 weeks in the first case and after 4 weeks in the second.

Bacterial growth in the host plant was quantified as described previously (Malamud *et al*., 2011). Briefly, bacteria were swabbed onto injured lemon leaves at a final concentration of 1×10^8 cfu/mL; four samples were taken for each strain at each time interval. Populations were quantified by macerating 1-cm^2 leaf discs in 0.5 mL of sterile water, followed by dilution plating onto PYM with the appropriate antibiotics. Plates were incubated at 28 °C, and colonies were counted after 48 h. Population data were transformed to $\log_{10}$ values, and standard errors were determined.

Cyclic β-1,2-glucan preparation

Xcc strains were grown overnight in 300 mL PYM. Cells were harvested by centrifugation at 10 000 ***g*** for 20 min and washed with 30 mM Tris-HCl buffer, pH 8. Cell pellets were extracted with 5% TCA to release periplasmic glucans, as described by Talaga *et al*. (1996). The cells were then pelleted and the supernants were concentrated by rotary evaporation. The supernatant samples were dissolved in 5% (v/v) acetic acid prior to size-exclusion chromatography on a BioGel P4 column (Biorad, Richmond, CA, USA) to determine cell-associated glucans. For BioGel P4 chromatography, a column (1.5×42 cm^2) was equilibrated and eluted with acetic acid 5% (v/v). Fractions (0.75 mL) were collected at a flow rate of 20 mL/h and assayed for carbohydrate using the anthrone reagent (Loewus, 1952). Fractions containing cyclic glucan were pooled and concentrated by rotary evaporation before being subjected to TLC. Samples were subjected to TLC on a silica gel plate in butan-1-ol : ethanol : water (5:5:4, v/v/v) with two developments. Carbohydrates were detected by spraying the TLC plate with a solution of 5% (v/v) sulphuric acid in ethanol, followed by heating at 120 °C. Photographs were obtained by scanning TLC (Vojnov *et al*., 2001).

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