Xanthomonas citri subsp. *citri* Type IV Pilus Is Required for Twitching Motility, Biofilm Development, and Adherence

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Submitted 21 March 2014. Accepted 28 June 2014.

Bacterial type IV pili (T4P) are long, flexible surface filaments that consist of helical polymers of mostly pilin subunits. Cycles of polymerization, attachment, and depolymerization mediate several pilus-dependent bacterial behaviors, including twitching motility, surface adhesion, pathogenicity, natural transformation, escape from immune system defense mechanisms, and biofilm formation. The *Xanthomonas citri* subsp. *citri* strain 306 genome codes for a large set of genes involved in T4P biogenesis and regulation and includes several pilin homologs. We show that *X. citri* subsp. *citri* can exhibit twitching motility in a manner similar to that observed in other bacteria such as *Pseudomonas aeruginosa* and *Xylella fastidiosa* and that this motility is abolished in *Xanthomonas citri* subsp. *citri* knockout strains in the genes coding for the major pilin subunit PilA_{XAC3241}, the ATPases PilB_{XAC3239} and PilT_{XAC2924}, and the T4P biogenesis regulators PilZ_{XAC1133} and FimX_{XAC2398}. Microscopy analyses were performed to compare patterns of bacterial migration in the wild-type and knockout strains and we observed that the formation of mushroom-like structures in *X. citri* subsp. *citri* biofilm requires a functional T4P. Finally, infection of *X. citri* subsp. *citri* cells by the bacteriophage ΦXacm4-11 is T4P dependent. The results of this study improve our understanding of how T4P influence *Xanthomonas* motility, biofilm formation, and susceptibility to phage infection.

Bacterial type IV pili or type IV fimbriae (T4P) are flexible surface filaments 4 to 7 nm in diameter and several micrometers in length that can resist more than 100 pN of stress force (Maier et al. 2002). T4P are involved in a variety of important bacterial functions, including twitching motility, surface adhesion, pathogenicity, natural transformation, immune escape, biofilm formation, and chemotaxis (Craig et al. 2004; Mattick 2002; Nudleman and Kaiser 2004). These structures have been most extensively studied in the genera *Pseudomonas*, *Neisseria*, and *Myxococcus* (Burrows 2012; Mattick 2002; Mauriello and

Zusman 2007; Merz and So 2000). In *Pseudomonas aeruginosa*, the filament of the T4P is composed of a helical polymer of pilin subunits, mostly PilA plus the minor pilins PilE, PilV, PilW, PilX, and FimU (Burrows 2012; Giltner et al. 2010; Mattick 2002), and its biogenesis and function are controlled by over 40 genes (Strom and Lory 1993).

Craig and associates (2004) proposed a generalized mechanism for T4P assembly–disassembly based on the T4P structure from *Neisseria gonorrhoeae* and similarities among the T4P from other organisms, including a conserved pilin subunit structural core, a left-handed three-start helical filament architecture, and conserved components of the pilus biogenesis apparatus. Prepilin monomers are processed in the inner membrane by the prepilin peptidase PilD before being incorporated into the base of the growing polymer in a process that requires the inner membrane integral protein PilC and the ATPase activity of PilB (Chiang et al. 2005). The growing pilus passes through the outer membrane secretin pore subcomplex formed by PilQ (Friedrich et al. 2014). Pilus retraction involves depolymerization at the pilus base and requires the ATPase activity of PilT (Chiang et al. 2008).

T4P are divided into two major subclasses, type IVa and IVb. These classes were first based on differences in the amino acid sequence and length of their major pilin subunits (Craig and Li 2008) but now has been extended to differences in the composition of the assembly apparatus (Burrows 2012). Type IVa pilins have a shorter leader sequence (5 to 6 amino acids) and a shorter mature sequence (approximately 150 amino acids) than type IVb pilins (15 to 30 amino acids and approximately 190 amino acids, respectively). The N-methylated N-terminal residue is phenylalanine for the type IVa pilins but varies for the type IVb pilins (Craig et al. 2004). Type IVa pilins are present in a broad range of bacteria, including plant and animal pathogens such as *Pseudomonas*, *Neisseria*, *Myxococcus*, *Shewanella*, and *Xanthomonas* spp. (Pelicic 2008; Tammam et al. 2011; Wall and Kaiser 1999), while type IVb pilins are found almost exclusively in enteric pathogens such as *Vibrio cholera* and enteropathogenic and enterotoxigenic *Escherichia coli* and *Salmonella enterica* (Craig and Li 2008; Faast et al. 1989). Both type IVa and IVb pilins have been associated with cell-to-cell aggregation and adherence to biotic and abiotic surfaces while only type IVa pilins mediate twitching motility (Burrows 2012).

Twitching motility is a flagella-independent form of bacterial translocation over moist organic and inorganic surfaces (Henrichsen 1983; Henrichsen et al. 1972). It occurs by the extension, tethering, and then retraction of polar T4P. Twitching motility is important in host colonization by a wide range of plant and animal pathogens (Comolli et al. 1999; Hazlett et al. 1991; Merz and So 2000; Nguyen et al. 2012; Singh et al. 2000;

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*The e-Xtra logo stands for “electronic extra” and indicates that six supplementary figures, six supplementary videos, and four supplementary tables are published online and that Figures 5, 6, 7, 8, 9, and 10 appear in color online.

Taguchi and Ichinose 2011), as well as in the formation of biofilm (Bahar et al. 2009; Li and Wang 2011; Varga et al. 2008). Some reports have shown that twitching motility is influenced by nutritional conditions, cell density, and soluble and cell contact-dependent intercellular signals (Huang et al. 2003; Mattick 2002). Twitching motility has been well described in some gram-negative bacteria such as *P. aeruginosa*, *N. gonorrhoeae*, and *Myxococcus xanthus*; however, the environmental signals that control twitching motility are not well understood. Although twitching has not yet been described in *Xanthomonas* spp., it has been observed in the related *Xylella* genus, where it was shown to be required for bacterial migration in the plant vessel. In *Xylella* spp., T4P also play a role in surface attachment, biofilm formation, and cell-to-cell aggregation (Caserta et al. 2010; De La Fuente et al. 2007; Li et al. 2007; Meng et al. 2005).

The first descriptions of T4P in *Xanthomonas* spp. were in *Xanthomonas campestris* pv. *hyacinthi* (van Doorn et al. 1994) and *X. campestris* pv. *vesicatoria* (Ojanen-Reuhs et al. 1997). However, very little is known regarding T4P function in *Xan-*

thomonas spp. except for the observation that T4P mediate the susceptibility of *X. citri* subsp. *citri* strain XW47 to infection by the filamentous phage Cf (Su et al. 1999; Yang et al. 2004). Like most other *Xanthomonas* spp. whose genomes have been sequenced, the genome of the citrus pathogen *X. citri* subsp. *citri* strain 306 (formerly *X. axonopodis* pv. *citri*) codes for all the components necessary for a functional T4P (da Silva et al. 2002) (Supplementary Table S1). The *X. citri* subsp. *citri* genes that code for products most similar to the *P. aeruginosa* major pilin PilA are *fimA*_{XAC3240} and *fimA*_{XAC3241}, with whom they share 40 and 43% identity, respectively, at the protein level. Therefore, we will refer to these *X. citri* subsp. *citri* gene products as PilA_{XAC3240} and PilA_{XAC3241}. PilA_{XAC3240} and PilA_{XAC3241} are 67.6% identical and show between 53 and 69% identity with homologs in other *Xanthomonas* spp., as well as the major pilin subunit from *Xylella fastidiosa* (Fig. 1A). As in *P. aeruginosa*, the *Xanthomonas citri* subsp. *citri* pilA genes are found in a gene cluster with the other T4P-related genes *pilR*, *pilS*, *pilB*, *pilC*, and *pilD* (Fig. 1B).

Fig. 1. A, Multiple protein alignment of PilA_{XAC3241} and PilA_{XAC3240} from *Xanthomonas citri* subsp. *citri* and their homologs from other selected *Xanthomonas* and a plant-associated *Pseudomonas* sp. The alignment was generated using CLUSTAL W (Larkin et al. 2007) with default parameters and processed with JalView (Waterhouse et al. 2009). The highly conserved G-1F+1E+5 motif that is required for both cleavage of the leader sequence and subsequent methylation of the mature protein is enclosed by boxes. Pilin sequence accession numbers: *X. citri* subsp. *citri* PilA_{XAC3241} (GenBank accession number AAM38085.1) and PilA_{XAC3240} (number AAM38084.1), *X. fragariae* (WP_002812271.1), *X. campestris* (number WP_010375211.1), *X. vesicatoria* (number WP_005992828.1), *X. axonopodis* pv. *citrumelo* F1 (number YP_004852685.1), *Stenotrophomonas maltophilia* (number WP_005418622.1), *S. maltophilia* JV3 (number YP_004793746.1), *Xylella fastidiosa* (number WP_020851240.1), *Xanthomonas campestris* pv. *campestris* (number NP_638446.1), and *Pseudomonas syringae* pv. *actinidiae* (number WP_020353487.1). **B**, Gene organization of the pilA genes. The direction of arrows indicates relative transcriptional orientation. The gray box indicates a putative rho-independent transcriptional terminator. RBS = ribosome binding site.

PilZ and FimX are two of the few regulators of T4P biogenesis that have been characterized. PilZ is a small protein required for twitching motility and T4P biogenesis in *P. aeruginosa* (Alm et al. 1996). Although PilZ does not bind *bis*(3'→5') cyclic dimeric guanosine monophosphate (c-di-GMP) (Guzzo et al. 2009), it belongs to a large family of proteins that are receptors for this bacterial second messenger (Amikam and Galperin 2006). FimX is a large protein with REC, PAS, GGDEF, and EAL domains that appear in that order in the FimX sequence. FimX is also required for T4P biogenesis in *P. aeruginosa* (Huang et al. 2003). Its N-terminal REC domain is required for FimX localization to the inner membrane in *P. aeruginosa* (Kazmierczak et al. 2006) and lacks a conserved aspartic acid residue required for phosphorylation by a cognate histidine kinase (Huang et al. 2003). The GGDEF and EAL domains, often associated with diguanylate cyclase and phosphodiesterase activities in other proteins (Simm et al. 2004), are inactive in FimX (Navarro et al. 2009; Newell et al. 2009). However, the FimX EAL domain retains its ability to bind c-di-GMP (Guzzo et al. 2013; Navarro et al. 2009). Although no interactions were detected between PilZ and FimX from *P. aeruginosa* (Qi et al. 2012), an interaction between their homologs in *X. citri* subsp. *citri* has been observed (Chin et al. 2012; Guzzo et al. 2009), and the PilZ-FimX (EAL domain)-c-diGMP complex from *X. citri* subsp. *citri* (Guzzo et al. 2013) and the related *X. campestris* pv. *campestris* strain 17 (Chin et al. 2012) have been crystallized. These differences in behavior of the *P. aeruginosa* and *Xanthomonas* PilZ and FimX proteins may be due to the relatively low sequence identity between PilZ (65%) and especially FimX (33%) homologs in *P. aeruginosa* and *X. citri* subsp. *citri* (Qi et al. 2012). Therefore, T4P biogenesis regulation by FimX and PilZ may act through different mechanisms in *P. aeruginosa* and in *X. citri* subsp. *citri*. In addition to its interactions with FimX, *X. citri* subsp. *citri* PilZ interacts with the hexameric ATPase PilB (Guzzo et al. 2009). These interactions point to a means by which specific signals that impinge on FimX could directly, or indirectly via PilZ, regulate PilB function in *X. citri* subsp. *citri* and related *Xanthomonas* spp.

Although FimX and PilZ have been shown to control T4P biogenesis and twitching motility in *P. aeruginosa* (Alm et al. 1996; Huang et al. 2003; Kazmierczak et al. 2006), T4P production and twitching motility have not been observed in *X. citri* subsp. *citri* or in other *Xanthomonas* spp. Thus far, PilB, PilZ, and FimX knockouts in *X. citri* subsp. *citri* have been shown to exhibit increased sliding motility, a type of movement that is independent of, and in some conditions inhibited by, T4P and flagella (Guzzo et al. 2009; Murray and Kazmierczak 2008). Therefore, the role of T4P in twitching motility and in other behavior of *X. citri* subsp. *citri* is poorly understood and the way that FimX and PilZ contribute to the regulation of T4P biogenesis in response to c-di-GMP is still unknown.

Here, we present the first demonstration of subsurface twitching motility in the genus *Xanthomonas*. We show that twitching motility in *X. citri* subsp. *citri* is dependent on the T4P components PilA, PilB, and PilT and the T4P regulators FimX and PilZ. We also show that T4P contribute to the formation of mushroom-like structures in *X. citri* subsp. *citri* biofilms and adherence to plant tissue, and are required for the infection of *X. citri* subsp. *citri* cells by the bacteriophage ΦXacm4-11.

RESULTS

Characterization of pilins from *X. citri* subsp. *citri*.

The genome of *X. citri* subsp. *citri* strain 306 has two genes (*xac3240* and *xac3241*) that code for type IV pilins next to the

pilB (*xac3239*), *pilC* (*xac3242*), and *pilD* (*xac3243*) genes (Fig. 1B). Although these two pilin genes were originally annotated as *fimA*, we prefer to name them *pilA*_{XAC3240} and *pilA*_{XAC3241}, consistent with the nomenclature of other T4P-related genes in the *X. citri* subsp. *citri* genome and, due to their homology, with the well-studied PilA proteins in other bacterial species. The amino acid sequences of PilA_{XAC3240} and PilA_{XAC3241} share 67.6% identity (Fig. 1A). Their sequence identity with orthologs from other *Xanthomonas* spp. ranges from 58 to 69% (Fig. 1A). They also show strong identity with the major type IV pilins from *Xylella fastidiosa* (53%) and *P. aeruginosa* (42%) (Fig. 1A). These sequence alignments show that the most conserved region corresponds approximately to the 50 amino acid residues that immediately follow the N-terminus prepin leader sequence that contains the G-1F+1/E+5 motif (Fig. 1A) that is required for both cleavage of the leader sequence and subsequent methylation of the N-terminal phenylalanine residue of the mature protein by the type IV prepin leader peptidase PilD (Lory and Strom 1997; Strom and Lory 1993). In the well-studied *P. aeruginosa* prepins, the leader peptides direct them to the periplasm and prevent their spontaneous polymerization (Lory and Strom 1997). Both PilA_{XAC3240} and PilA_{XAC3241} contain the above elements necessary for their correct processing. In addition to *pilA*_{XAC3240} and *pilA*_{XAC3241}, the *Xanthomonas citri* subsp. *citri* genome codes for homologs of other pilin-like proteins that have been characterized in other bacteria (for example, the proteins XAC2664 to XAC2668 code for homologs of the *P. aeruginosa* pseudopilins PilE, PilY1, PilX, PilW, and PilV, respectively). Finally, the *xac3805* gene codes for another pilin-like protein that was originally annotated as PilA when the *X. citri* subsp. *citri* genome was sequenced (da Silva et al. 2002) and whose homolog has been implicated in *X. campestris* pv. *campestris* motility on Eiken agar (Ryan et al. 2007). However, a close analysis of the coding sequence reveals that the gene product is most likely not a bona fide type IVa pilin because i) it lacks the prepin leader motifs required for correct processing and ii) the annotated GTG start codon is preceded by 106 in-frame codons that are well conserved (though often overlooked during annotation) in XAC3805 homologs from several Xanthomonadaceae species (Supplementary Fig. S1). The N-terminal half of this extension (approximately 50 amino acids) corresponds to a domain of unknown function (Pfam DUF4339) conserved in bacteria, archaea, and eukaryotes (Finn et al. 2014).

In order to determine whether pilins are synthesized and secreted in *X. citri* subsp. *citri*, we raised polyclonal antibodies by immunizing rabbits with recombinant PilA_{XAC3240_37-151}. This PilA_{XAC3240} fragment lacks the highly hydrophobic first 36 residues while the full-length recombinant PilA_{XAC 3241} (residues 1 to 146) contains this region. These antibodies recognize both recombinant PilA_{XAC3240_37-151} (13.9 kDa) and PilA_{XAC3241_1-146} (15.3 kDa) (Supplementary Fig. S2), as expected from the high degree of sequence identity (above). We then used these antibodies to detect PilA_{XAC3240} or PilA_{XAC3241} in total cell lysates of wild-type *X. citri* subsp. *citri* and in the Δ *pilA*_{XAC3241}, Δ *pilB*_{XAC3239}, and Δ *pilZ*_{XAC1133} knockout strains. The results indicate that PilA_{XAC3240} or PilA_{XAC3241} are synthesized in the wild type and the Δ *pilB* and Δ *pilZ* knockout strains (Fig. 2). However, no immunoreactive protein is produced by the Δ *pilA*_{XAC3241} strain. Because the antibody recognizes both PilA_{XAC3240} and PilA_{XAC3241}, this result indicates that, under the conditions tested, Δ *pilA*_{XAC3241} is expressed at levels much greater than that of PilA_{XAC3240} (assuming the absence of polar effects caused by the in-frame deletion of a large fraction of the *pilA*_{XAC3241} coding sequence). These results are consistent with those previously reported for the human pathogen *P. aeruginosa*, where cellular pools of PilA are not modified in

ΔpilB and *ΔpilZ* strains (Alm et al. 1996; Huang et al. 2003; Turner et al. 1993).

We then used the anti-PilA antibodies to detect extracellular PilA. After growth on nutrient agar plates, *X. citri* subsp. *citri* cells were submitted to a shearing force that liberates extracellular pili. Extracellular PilA could be detected in the wild-type *X. citri* subsp. *citri* culture but was not detected in *ΔpilA*_{XAC3241} cells (Fig. 2B). Extracellular PilA was also not detected in the *ΔpilB* and *ΔpilZ* knockout strains (Fig. 2B). However, low levels of extracellular PilA could be detected in the *ΔpilZ* knockout strain carrying a plasmid that carries the *X. citri* subsp. *citri* *pilZ*_{XAC1133} gene (Fig. 2B). We note that, in *X. citri* subsp. *citri*, the *pilZ*₁₁₃₃ gene is found at the end of a putative polycistronic operon and, therefore, differences in gene expression and regulation could explain why we do not observe wild-type levels when PilZ is expressed ectopically. In the *ΔpilT* knockout strain, extracellular PilA was observed at levels significantly greater than in the wild-type strain (Fig. 2C), as has been observed for *ΔpilT* mutants in *Azoarcus* sp. strain BH72 (Bohm et al. 2007). Extracellular PilA dropped to close to wild-type levels in the *ΔpilT* strain expressing wild-type PilT (Fig. 2C).

We then performed a transcriptional analysis to compare the expression levels of *pilA*_{XAC3241} and *pilA*_{XAC3240} in *X. citri* subsp. *citri* cells growing in liquid medium (exponential growth in Luria Bertani [LB] medium) and growing on the plant surface (epiphytic growth) and within the mesophyll space (in planta growth) of orange leaves. We also analyzed the expression of the *xac3805* gene mentioned above. For epiphytic growth, the *X. citri* subsp. *citri* culture was sprayed onto orange leaf surfaces whereas, for in planta growth, plants were inoculated with *X. citri* subsp. *citri* cells by infiltration using a needleless syringe. After the indicated period of time, bacteria were isolated from the plant tissue, and RNA was extracted and subjected to quantitative real-time reverse-transcription polymerase chain reaction (RT-PCR) analysis. We observed that *pilA*_{XAC3241} was expressed at significantly greater levels than *pilA*_{XAC3240} and *xac3805* in liquid culture and at all timepoints after infiltration into the host tissue (Fig. 3A) and in *X. citri* subsp. *citri* cells grown on the surface of orange leaves (Fig. 3B). The *pilA*_{XAC3241}/*pilA*_{XAC3240} transcript ratio is significantly greater in *X. citri* subsp. *citri* cells upon infiltration into plant tissue when compared with the ratio in liquid culture and epiphytically grown cells (Fig. 3C). The two neighboring *pilA* genes, *pilA*_{XAC3240} and *pilA*_{XAC3241}, are separated by a palindromic sequence that could form a hairpin loop structure (Fig. 1B) and function as a transcriptional terminator, thereby explaining the large difference in expression levels observed for the two transcripts in *X. citri* subsp. *citri* cells grown in liquid media, on leaf surfaces, and within the host tissue (Fig. 3). Similar observations were reported for the two neighboring open reading frames (*fimA* and *fimB*) that code for PilA homologs in the related pathogen *X. campestris* pv. *vesicatoria* (Ojanen-Reuhs et al. 1997).

Role of the T4P in *X. citri* subsp. *citri* motility.

Most bacteria are able to move through liquids or across surfaces in search of nutrients or during the construction of complex multicellular biofilm structures (Costerton et al. 1995; Gloag et al. 2013; Klausen et al. 2003). Swimming and swarming motility are correlated with the presence of flagella while twitching motility across solid surfaces is dependent on T4P (Harshey 2003). We have previously shown that *X. citri* subsp. *citri* cultures can spread on semisolid agar surfaces by way of sliding motility, a mechanism independent of both flagella and T4P. Sliding motility seems to be inhibited by these bacterial surface structures because spreading is observed to increase in

the *X. citri* subsp. *citri* *ΔpilZ*, *ΔpilB*, and *ΔfimX* mutant strains and is not reduced in the *ΔfliC* (flagellin) knockout strain (Guzzo et al. 2009) (Supplementary Fig. S3). Increased sliding motility is also observed in the *ΔpilA*_{XAC3241} strain and motility reverts to wild-type levels in the *ΔpilA*_{XAC3241} strain carrying a plasmid for ectopic expression of PilA_{XAC3241}. The same phenomenon has been observed in a *P. aeruginosa* *ΔpilA* strain (Murray and Kazmierczak 2008).

To test whether increased sliding motility is due to increased exopolysaccharide production by the mutant strains, we measured extracellular polysaccharide (EPS) in liquid cultures of these strains as well as in a *ΔgumD* mutant that lacks a gene essential for xanthan gum production (Dunger et al. 2007). Although the *ΔgumD* strain had significantly reduced levels of EPS, the *ΔpilA*, *ΔpilB*, *ΔpilZ*, and *ΔfimX* strains all produced EPS levels similar to that of the wild-type strain (Supplementary Fig. S5). It may be that the increased sliding motility in *X. citri* is due to the secretion of other surfactants that are not

Fig. 2. Western blot analysis of PilA expression in *Xanthomonas citri* subsp. *citri* strains. **A**, Western blot of whole-cell proteins from *X. citri* subsp. *citri* wild type (Xac WT), *ΔpilA*_{XAC3241}, *ΔpilA*_{XAC3241} + pUFR*pilA*_{XAC3241} (*ΔpilAc*), *ΔpilB*_{XAC3239}, *ΔpilZ*_{XAC1133}, and *ΔpilZ*_{XAC1133} + pUFR *pilZ*_{XAC1133} (*ΔpilZc*). Polyclonal anti-PilA antibodies were used for detection. **B**, Western blot of extracellular PilA from Xac WT, *ΔpilA*_{XAC3241}, *ΔpilA*_{XAC3241} + pUFR*pilA*_{XAC3241} (*ΔpilAc*), *ΔpilB*_{XAC3239}, *ΔpilZ*_{XAC1133}, and *ΔpilZ*_{XAC1133} + pUFR *pilZ*_{XAC1133} (*ΔpilZc*). **C**, Western blot of extracellular PilA from Xac WT, *ΔpilT*_{XAC2924}, and *ΔpilT*_{XAC2924} + pUFR *pilT*_{XAC2924} (*ΔpilTc*). M = Positions and molecular weights of the PilA_{XAC3240} and full-length PilA_{XAC3241} bands. PilA_{XAC3240_37-151} is the recombinant PilA_{XAC3240} fragment protein which was used to produce the polyclonal antibodies that cross-react equally with both PilA_{XAC3240} and PilA_{XAC3241}.

polysaccharides. For example rhamnolipids have been shown to enhance *P. aeruginosa* sliding and swarming motility on soft agar surfaces (Caiazza et al. 2005; Deziel et al. 2003; Murray and Kazmierczak 2008).

Twitching motility is a mechanism of movement that is dependent on T4P and independent of flagella (Burrows 2012; Henriksen 1983; Mattick 2002; Merz and So 2000; Skerker and Berg 2001; Wall and Kaiser 1999). Twitching motility by some bacteria can be observed at the interstitial surface between nutrient agar and plastic or glass surfaces and is commonly used to test T4P function in *P. aeruginosa* (Beatson et al. 2002; Nolan et al. 2012; Semmler et al. 2000) and in *N. meningitidis* (Oldfield et al. 2007). We have identified conditions in which *X. citri* subsp. *citri* wild-type cells could be observed to migrate across a plastic surface under 1% agar (Fig. 4A). The assay was carried out by using two different media, Silva Buddenhagen (SB) and King's Broth (KB), that contain sucrose and glycerol, respectively, as their principal carbon sources (Fig. 4B and 4C). This migration is relatively slow (less than 1 cm over 3 to 5 days) compared with that commonly reported for *P. aeruginosa* (greater than 1 cm over 24 h) (Burrows 2012). Interestingly, we did not observe significant subsurface migration by the *X. campestris* pv. *campestris* strain ATCC33913 in KB medium (Fig. 4A). To determine that *X. citri* subsp. *citri* subsurface migration was, indeed, dependent upon T4P, we compared the wild-type

migration with that of *X. citri* subsp. *citri* mutant strains. In both media, the $\Delta pilA_{XAC3241}$, $\Delta pilB_{XAC3239}$, $\Delta pilZ_{XAC1133}$, $\Delta pilT_{XAC2924}$, and $\Delta fimX_{XAC2398}$ mutant *X. citri* subsp. *citri* strains all had reduced subsurface twitching motility when compared with the wild type (Fig. 4). Twitching motility of the $\Delta pilA_{XAC3241}$, $\Delta pilZ_{XAC1133}$, $\Delta pilT_{XAC2924}$, and $\Delta fimX_{XAC2398}$ strains could be restored to wild-type levels by the expression of the $pilA_{XAC3241}$, $pilZ_{XAC1133}$, $pilT_{XAC2924}$, and $fimX_{XAC2398}$ genes carried on ectopic plasmids (Fig. 4).

We then used fluorescence microscopy to analyze the multicellular organization at the edges of the subsurface twitching zones of *X. citri* subsp. *citri* cells containing a plasmid that expresses green fluorescent protein (GFP). The edge of the twitching zone of wild-type *X. citri* subsp. *citri* is made up of groups or islands of cells that seem to have separated from the main body of the colony (Fig. 5). These groups diminish in size the further they are from the main body until even individual isolated cells can be observed at the extreme edge of the colony (Fig. 5). As a result, the fringe of the wild-type *X. citri* subsp. *citri* colony undergoing twitching has a disaggregated aspect, with a poorly defined and irregular boundary line. On the other hand, the $\Delta pilA_{XAC3241}$, $\Delta pilB_{XAC3239}$, $\Delta pilZ_{XAC1133}$, and $\Delta fimX_{XAC2398}$ strains all present a smoother and more uniform and well-defined boundary, with tightly packed cells (Fig. 5). The roughness and irregular aspect of the twitching zone is

Fig. 3. $pilA_{XAC3240}$, $pilA_{XAC3241}$, and $xac3805$ transcript levels during the interaction of *Xanthomonas citri* subsp. *citri* with the orange host plant. Gene expression was analyzed by real time reverse-transcription polymerase chain reaction (SYBR system) at the indicated times after inoculation with *X. citri* subsp. *citri* at 1×10^7 CFU/ml. **A**, Inoculation in planta via infiltration of leaves with the *X. citri* subsp. *citri* culture using a syringe without a needle. **B**, Epiphytic inoculation by spraying the *X. citri* subsp. *citri* culture onto the abaxial surface of the leaf. **C**, The $pilA_{XAC3241}/pilA_{XAC3240}$ expression ratio at different times postinoculation using the two methods shown in parts A and B. Relative expression levels were calculated using the $2^{-\Delta\Delta CT}$ method. The zero time point corresponds to the transcript levels measured in liquid culture prior to inoculation. All transcript levels were corrected according to variations in the measurements of the constitutively expressed 16S $rRNA_{XAC4291}$ and $gyrA_{XAC1631}$ transcripts and normalized with respect to the $pilA_{XAC3240}$ transcript in liquid culture.

restored in the $\Delta pilA_{XAC3241}$ strain carrying a plasmid expressing the $pilA_{XAC3241}$ gene, in the $\Delta pilZ_{XAC1133}$ strain carrying a plasmid expressing the $pilZ_{XAC1133}$ gene, and in the $\Delta fimX_{XAC2398}$ strain carrying a plasmid expressing the $fimX_{XAC2398}$ gene (Fig. 5). To better visualize this phenomenon, time-lapse films showed that individual wild-type *X. citri* subsp. *citri* cells at the edge of the subsurface twitching zone undergo jerky twitching motions (Supplementary Video S1). These twitching motions were not observed for T4P knockout *X. citri* subsp. *citri* strains (Supplementary Videos S2 to S5) but reappeared in the $\Delta pilZ_{XAC1133}$ strain complemented with a plasmid carrying the wild-type $pilZ_{XAC1133}$ gene (Supplementary Video S6).

An analysis of a large number of tracts of the edges of the subsurface twitching zones of the $\Delta pilA_{XAC3241}$, $\Delta pilB_{XAC3239}$, $\Delta pilZ_{XAC1133}$, and $\Delta fimX_{XAC2398}$ strains revealed that, occasionally, the linear nature of the colony edge is interrupted by dozens or a few hundred cells at various stages of a process in which they appear to be budding from the main body of the colony (Fig. 6 for the $\Delta pilA_{XAC3241}$ mutant and data not shown for the $\Delta pilB_{XAC3239}$, $\Delta pilZ_{XAC1133}$, and $\Delta fimX_{XAC2398}$ mutants).

These smooth protuberances are only observed in the T4P mutant strains but not in the wild-type strain (data not shown). We then asked whether wild-type and mutant cells could interact together at the twitching border or whether they would tend to segregate. To test this, we mixed wild-type and mutant *X. citri* subsp. *citri* cells expressing either GFP or cyan fluorescent protein (CFP). Fluorescence microscopy images of the twitching border zones of these mixed cell populations are shown in Figure 7. When wild-type-GFP cells were mixed with $\Delta pilA_{XAC3241}$ -CFP, $\Delta pilZ_{XAC1133}$ -CFP, $\Delta pilB_{XAC3239}$ -CFP, or $\Delta fimX_{XAC2398}$ -CFP cells, the two cell populations remained segregated, with the T4P mutant cells forming dense aggregates and the wild-type cells forming much smaller aggregates and sometimes seen as individual cells. Interestingly, the more dispersed wild-type cells seemed to be above the more densely packed mutant cells (Fig. 7). When wild-type-GFP and wild-type-CFP cells were mixed, the two cell populations mixed to a significant degree in a dispersed manner at the twitching border zone. This dispersion and mixing of populations was also observed for the mixture of wild-type-GFP cells with

Fig. 4. Type IV pili from *Xanthomonas citri* subsp. *citri* are essential for twitching subsurface motility. **A**, Twitching subsurface motility assays in plates containing King's Broth agar (1% wt/vol) medium supplemented with 2 mM $CaCl_2$. Nutrient agar was removed 5 days after inoculation. The twitching zone was visualized by staining with crystal violet. Xac WT = *X. citri* subsp. *citri* wild type, Xcc = *X. campestris* pv. *campestris*, $\Delta fliC$ = $\Delta fliC$ knockout, $\Delta pilA$ = $\Delta pilA_{XAC3241}$ knockout, $\Delta pilAc$ = $\Delta pilA_{XAC3241}$ knockout complemented with pUFRpilA_{XAC3241}, $\Delta pilB$ = $\Delta pilB_{XAC3239}$ knockout, $\Delta pilZ$ = $\Delta pilZ_{XAC1133}$ knockout, $\Delta pilZc$ = $\Delta pilZ$ knockout complemented with pUFRpilZ, $\Delta fimX$ = $\Delta fimX_{XAC2398}$ knockout, $\Delta fimXc$ = $\Delta fimX$ knockout complemented with pUFRfimX, $\Delta pilT$ = $\Delta pilT_{XAC2924}$ knockout, and $\Delta pilTc$ = $\Delta pilT$ knockout complemented with pUFRpilT. The black bar corresponds to 5 mm. **B**, Diameters of the twitching zones in experiments performed as described in part A. **C**, Diameter of twitching zones of experiments performed as in part A, except that the Silva Buddenhagen agar (1% wt/vol) medium was used. In B and C, the mean diameter of the subsurface twitching zone was measured. Experiments were repeated five times (each time in duplicate). Vertical bars represent the standard errors of the means.

$\Delta pilA_{XAC3241}$ -CFP, $\Delta pilZ_{XAC1133}$ -CFP, or $\Delta fimX_{XAC2398}$ -CFP cells complemented with a plasmid carrying the $pilA_{XAC3241}$, $pilZ_{XAC1133}$, or $fimX_{XAC2398}$ gene, respectively (Fig. 7).

The *Xanthomonas* PilZ and FimX proteins have been shown to interact with each other (Guzzo et al. 2009). In *X. citri* subsp. *citri*, the PilZ–FimX_{EAL} interaction involves conserved PilZ_{XAC1133} residues that localize on a specific region of the protein surface (Guzzo et al. 2009, 2013). In addition to its interactions with FimX, PilZ also interacts with the PilB ATPase (Guzzo et al. 2009). Microscopic images of the twitching zones of *X. citri* subsp. *citri* $\Delta pilZ_{XAC1133}$ cells containing plasmids expressing a set of PilZ_{XAC1133} mutants are shown in Figure 8. Although *X. citri* subsp. *citri* expressing the PilZ Y25A, F28A, and E101A mutants presented an irregular twitching border, with many individual cells or small groups of cells, the W69A and L104A mutants displayed a tighter, more organized twitching border, with very few free cells visible. Furthermore, the *X. citri* subsp. *citri* $pilZ_{XAC1133}$ cells contain-

ing a plasmid expressing PilZ_{XAC1133} protein lacking the C-terminal 11 amino acids (residues 107 to 117) also presented a highly organized twitching border, with few individual cells (Fig. 8). Although these C-terminal residues are well conserved in the group of homologs most closely related to *Xanthomonas* and *Pseudomonas* PilZ T4P regulators, they are not required for the interaction with FimX but, rather, seem to take part in PilZ's interaction with PilB (Guzzo et al. 2009).

The T4P contributes to the formation of specialized mature structures in *X. citri* subsp. *citri* biofilm.

Bacterial biofilms are thin layers of cells adhering to a surface, together with secreted polymers. In this sense, subsurface twitching zones can, in some ways, be considered biofilms in motion. However, the thickness and other structural features of the biofilm in a subsurface twitching zone may be restricted by the physical forces applied to the bacteria at the plastic–agar interface. In order to observe other possible aspects of *X. citri*

Fig. 5. *Xanthomonas citri* subsp. *citri* type IV pili knockouts have a compact migration front. *X. citri* subsp. *citri* strains were stab inoculated through a thin layer of King's Broth agar (1% wt/vol) supplemented with 2 mM CaCl₂ and incubated at 28°C for 2 days. Bacteria were observed by confocal microscopy at $\times 100$ magnification. Images show the fringes of the twitching zones at the interstitial surfaces between the glass base of the microscopy chamber and nutrient-agar medium of *X. citri* subsp. *citri* wild type (Xac WT), $\Delta pilA_{XAC3241}$, $\Delta pilA_{XAC3241}$ + pUFRpilA_{XAC3241} ($\Delta pilAc$), $\Delta pilB_{XAC3239}$, $\Delta pilZ_{XAC1133}$, and $\Delta fimX_{XAC2398}$ strains carrying pBBR-5GFP and of $\Delta pilZ_{XAC1133}$ + pUFRpilZ ($\Delta pilZc$) and $\Delta fimX_{XAC2398}$ + pUFRfimX ($\Delta fimXc$) strains carrying pBBR-2GFP. Left panels show the three-dimensional (3D) reconstructed images; right panels show the x-y plane.

subsp. *citri* biofilm structure that may depend on T4P, we used fluorescence microscopy to analyze the mature structure of *X. citri* subsp. *citri* biofilm grown on a glass surface under liquid nutrient medium. *X. citri* subsp. *citri* wild-type cells produce a

thicker biofilm (approximately 20 μ m) than those produced by the $\Delta pilA_{XAC3241}$, $\Delta pilZ_{XAC1133}$, $\Delta pilB_{XAC3239}$ and $\Delta fimX_{XAC2398}$ mutants (approximately 8 μ m) (Fig. 9; Supplementary Fig. S4). The $\Delta pilA_{XAC3241}$, $\Delta pilZ_{XAC1133}$, and $\Delta fimX_{XAC2398}$ strains

Fig. 6. A, Representative images of the fringe of a subsurface colony of *Xanthomonas citri* subsp. *citri* $\Delta pilA_{XAC3241}$ + pBBR-5GFP growing at the interstitial surface between the glass base of a microscopy chamber and King's Broth agar (1% wt/vol) + 2 mM $CaCl_2$ at 28°C for 3 days. Images were obtained using an inverted fluorescence microscope at $\times 100$ magnification. **B,** Schematic representation from the images taken in A.

Fig. 8. $PilZ_{W69A}$, $PilZ_{L104A}$, and $PilZ_{\Delta 107-117}$ mutants have impaired twitching motility. Subsurface twitching assay of *Xanthomonas citri* subsp. *citri* $\Delta pilZ_{XAC1133}$ complemented with the indicated *PilZ* mutants ($PilZ_{Y25A}$, $PilZ_{F28A}$, $PilZ_{W69A}$, $PilZ_{E101A}$, $PilZ_{L104A}$, and $PilZ_{\Delta 107-117}$). Images were taken by using a fluorescence microscope with a magnification power of $\times 100$. *PilZ* mutants and wild-type *X. citri* subsp. *citri* carrying pBBR-5GFP were stab inoculated through a thin layer of King's Broth medium supplemented with 1% (wt/vol) agar and 2 mM $CaCl_2$ and incubated at 28°C for 2 days.

Fig. 7. Images of the fringes of the subsurface twitching zones of co-cultures of *Xanthomonas citri* subsp. *citri* wild-type and mutant strains. *X. citri* subsp. *citri* strains were transformed with pBBR-5GFP for expression of green fluorescent protein (GFP; green) or with pBBR-5CFP or pBBR-2CFP for expression of cyan fluorescent protein (CFP; blue), as indicated. Knockouts and wild-type *X. citri* subsp. *citri* were stab inoculated through a thin layer of King's Broth medium supplemented with 1% (wt/vol) agar and 2 mM $CaCl_2$ and incubated at 28°C for 2 days. Fringes of the twitching zones at the interstitial surfaces between the glass base of a microscopy chamber and nutrient medium of mixtures of *X. citri* subsp. *citri* wild type (Xac WT) and the *X. citri* subsp. *citri* knockout strains ($\Delta pilA_{XAC3241}$, $\Delta pilB_{XAC3239}$, $\Delta pilZ_{XAC1133}$, and $\Delta fimX_{XAC2398}$ carrying pBBR-5CFP; and $\Delta pilAc$: $\Delta pilA_{XAC3241}$ + pUFRpilA_{XAC3241}, $\Delta pilZc$: $\Delta pilZ_{XAC1133}$ + pUFRpilZ, and $\Delta fimXc$: $\Delta fimX_{XAC2398}$ + pUFRfimX carrying pBBR-2CFP) were observed by using a fluorescence microscope with a magnification power of $\times 100$.

complemented with a plasmid expressing $PilA_{XAC3241}$, $PilZ_{XAC1133}$, or $FimX_{XAC2398}$, respectively, present biofilms with thickness similar to that of the wild-type strain (Fig. 9). The wild-type biofilm is made up of many three-dimensional, columnar or mushroom-like structures whereas these structures are largely absent in the biofilms formed by the $\Delta pilA_{XAC3241}$, $\Delta pilZ_{XAC1133}$, $\Delta pilB_{XAC3239}$, and $\Delta fimX_{XAC2398}$ mutants (Fig. 10). Instead, the mutant cells form a thinner, more homogeneous and more closely packed biofilm layer (Fig. 10). The *X. citri* subsp. *citri* $\Delta pilA_{XAC3241}$, $\Delta pilZ_{XAC1133}$, and $\Delta fimX_{XAC2398}$ strains expressing wild-type $PilA_{XAC3241}$, $PilZ_{XAC1133}$, or $FimX_{XAC2398}$, respectively, produce biofilms similar to those of the wild-type strain (Fig. 10).

T4P of *X. citri* subsp. *citri* participate in the adherence to the host plant.

T4P have been shown to play a role in the adhesion of bacteria to surfaces (Bahar et al. 2010; Kang et al. 2002; Ramphal

et al. 1984; Rudel et al. 1992; Winther-Larsen et al. 2001). Therefore, we decided to evaluate the participation of the T4P in the ability of *X. citri* subsp. *citri* cells to adhere to orange plants. Liquid *X. citri* subsp. *citri* cultures were applied to citrus leaves and allowed to interact for 6 h, after which the quantity of adhered cells was evaluated. We observed that $\Delta pilA_{XAC3241}$, $\Delta pilB_{XAC3239}$, $\Delta pilZ_{XAC1133}$, and $\Delta fimX_{XAC2398}$ mutant *X. citri* subsp. *citri* strains have significantly reduced adherence to the host plant when compared with wild-type *X. citri* subsp. *citri* (Fig. 11). *X. citri* subsp. *citri* $\Delta pilA_{XAC3241}$, $\Delta pilZ_{XAC1133}$, and $\Delta fimX_{XAC2398}$ strains expressing wild-type $PilA_{XAC3241}$, $PilZ_{XAC1133}$, or $FimX_{XAC2398}$, respectively, have adherence similar to those of the wild-type *X. citri* subsp. *citri* strain (Fig. 11).

Because the *X. citri* subsp. *citri* T4P mutants all present altered phenotypes in biofilm, twitching, and plant adherence assays, we next asked whether these cells were compromised in their ability to cause citrus canker symptoms. The $\Delta pilA_{XAC3241}$, $\Delta pilZ_{XAC1133}$, $\Delta pilB_{XAC3239}$, and $\Delta fimX_{XAC2398}$ mutants are capable

Fig. 9. Type IV pili knockouts *Xanthomonas citri* subsp. *citri* have a disrupted biofilm. Biofilm from *X. citri* subsp. *citri* wild type (Xac WT), $\Delta pilA_{XAC3241}$, $\Delta pilB_{XAC3239}$, $\Delta pilZ_{XAC1133}$, and $\Delta fimX_{XAC2398}$ carrying pBBR-5GFP and $\Delta pilA_{XAC3241}$ + pUFRpilA_{XAC3241} ($\Delta pilAc$), $\Delta pilZ_{XAC1133}$ + pUFRpilZ ($\Delta pilZc$), and $\Delta fimX_{XAC2398}$ + pUFRfimX ($\Delta fimXc$) carrying pBBR-2GFP were observed by using confocal microscopy with a magnification power of $\times 40$. *X. citri* subsp. *citri* strains were inoculated in King's broth medium supplemented with 2% (wt/vol) glucose and incubated in a microscopy chamber at 28°C for 5 days.

of producing citrus canker lesions 7 days after infiltration into citrus leaves, in a manner very similar to that observed for wild-type *X. citri* subsp. *citri* cells (Supplementary Fig. S6A). In addition, no differences were observed in the number of viable cells that could be retrieved from these lesions up to 16 days postinoculation. Finally, we asked whether the T4P contributes to *X. citri* subsp. *citri* survival when grown on the surface of citrus leaves. There was no significant difference between the *X. citri* subsp. *citri* wild-type and $\Delta pilA_{XAC3241}$ strains in the number of bacterial cells recovered up to 15 days after spraying onto citrus leaf surfaces.

***X. citri* subsp. *citri* T4P are required for infection by bacteriophage $\Phi Xacm4-11$.**

Infection of bacteria by bacteriophages often requires specific piliated structures on the bacterial surface. For example, *E. coli* infection by the R17 phage requires a mating pilus, related to type IV secretion systems (T4SS) (Berry and Christie 2011). On the other hand, *P. aeruginosa* PO1 infection by the ΦKMV phage and *P. aeruginosa* PAK infection by the PO4 phage requires a T4P (Alm et al. 1996; Chibeu et al. 2009). A few groups have studied the infection of *Xanthomonas* spp. by bacteriophages (Ahern et al. 2014; Ahmad et al. 2014; Balogh et al. 2013; Dai et al. 1987; Hung et al. 2003; Kuo et al. 1987, 1994; Lee et al. 2001; Lee et al. 2009; Lin et al. 1994; Su et al. 1999; Yang et al. 2004; Yuzenkova et al. 2003). In one case, infection of an *X. citri* strain by the filamentous phage cf was abolished in

a *pilA* knockout strain (Su et al. 1999) and in a strain lacking PilR, a transcriptional regulator of the *pilA* gene (Yang et al. 2004). Recently, Ahern and associates (2014) showed that the infection of *X. fastidiosa* strain Temecula and the *Xanthomonas* sp. rice isolate EC-12 by the siphophages Sano and Salvo and podophages Prado and Paz is dependent on the *pilA* gene.

$\Phi Xacm4-11$ is a bacteriophage isolated from *Xanthomonas* spp. in Florida that has been shown to be able to infect the *X. citri* subsp. *citri* strain 306 used in this study (Balogh et al. 2013). We then asked whether this infection process was dependent on the *X. citri* subsp. *citri* T4P. Both wild-type *X. citri* subsp. *citri* cells and a *X. citri* subsp. *citri* strain with a deletion in the *virB7*_{XAC2622} gene, which codes for a subunit of the *X. citri* subsp. *citri* T4SS (Alegria et al. 2005; Souza et al. 2011), are susceptible to infection by $\Phi Xacm4-11$ (Fig. 12). However, the *X. citri* subsp. *citri* $\Delta pilA_{XAC3241}$, $\Delta pilB_{XAC3239}$, $\Delta pilZ_{XAC1133}$, and $\Delta fimX_{XAC2398}$ mutants are resistant to infection, indicating that T4P play a role in this process. Complementation of the $\Delta pilA_{XAC3241}$, $\Delta pilZ_{XAC1133}$, and $\Delta fimX_{XAC2398}$ mutants with the ectopic *pilA*_{XAC3241}, *pilB*_{XAC3239}, *pilZ*_{XAC1133}, and *fimX*_{XAC2398} genes restored *X. citri* subsp. *citri* susceptibility to phage infection (Fig. 12). The $\Delta pilT_{XAC2924}$ mutant was also resistant to $\Phi Xacm4-11$ infection and susceptibility was restored in the mutant cell line carrying an ectopic *pilT*_{XAC2924} gene (Fig. 12A). Because *Pseudomonas* spp. $\Delta pilT$ mutants are hyperpiliated but deficient in pilus retraction (Bertrand et al. 2010; Graupner et al. 2001; Whitchurch and Mattick 1994), these

Fig. 10. Three-dimensional (3D) reconstruction of biofilm from *Xanthomonas citri* subsp. *citri*. Type IV pili knockouts and wild-type *X. citri* subsp. *citri* (Xac WT) expressing green fluorescent protein (GFP) were grown for 5 days at 28°C in King's Broth medium supplemented with 2% (wt/vol) glucose into chambered microscope slides. Images were taken with a confocal microscope at $\times 100$ magnification and 3D reconstructions were carried out by using Volume Viewer from Fiji (ImageJ) software.

results suggest that pilus retraction is required for Φ Xacm4-11 infection of *X. citri* subsp. *citri*.

We also tested the ability of Φ Xacm4-11 to infect *X. citri* subsp. *citri* Δ *pilZ*_{XAC1133} mutant cells containing plasmids expressing the *PilZ*_{XAC1133} Y25A, F28A, W69A, E101A, L104A, and Δ 107-117 mutants. We observed that the mutant *X. citri* subsp. *citri* strain expressing the *PilZ* Y25A, F28A, and E101A mutants were infected by the phage Φ Xacm4-11, while W69A, L104A, and Δ 107-117 mutants were resistant to the phage

Fig. 11. *Xanthomonas citri* subsp. *citri* knockouts in pilus components have impaired adherence to citrus leaves. Aliquots (20 μ l) of bacterial culture (1×10^7 CFU/ml) were spotted onto citrus abaxial leaf surfaces and incubated for 6 h at 28°C in a humid chamber. Bacterial adherence was quantified by crystal violet staining. Experiments were repeated three times with four replicates each time. Vertical bars represent the standard errors of the means.

infection (Fig. 12A). These results, together with the twitching assay results (Fig. 8), suggest that T4P biogenesis or function is severely impaired in the *PilZ* W69A, L104A, and Δ 107-117 mutants.

DISCUSSION

The *X. citri* subsp. *citri* genome codes for pilins and pilus assembly proteins characteristic of type IVa pili (Burrows 2012). A few studies have addressed some aspects of pilin gene expression and T4P production in *Xanthomonas* spp. (Ojanen-Reuhs et al. 1997; Su et al. 1999; van Doorn et al. 1994; Yang et al. 2004), and Ahern and associates (2014) recently used bacterial colony morphology as an indicator of twitching motility in *X. fastidiosa* and *Xanthomonas* EC-12. The present study is the first report of subsurface T4P-dependent twitching motility in *Xanthomonas* spp. and the first investigation of the role of T4P in the formation of *Xanthomonas* biofilms. The process of bacterial biofilm formation is characterized by five stages: reversible attachment, irreversible attachment, microcolony formation, three-dimensional structure formation, and, finally, cell detachment and dispersion from the biofilm (Renner and Weibel 2011), in which cell-to-cell signaling or quorum sensing plays an important role (Davies et al. 1998; Dow et al. 2003). Biofilm formation begins with the reversible attachment of cells to either an organic or inorganic surface using extracellular organelles such as flagella, T4P, and other fimbriae (Bullitt and Makowski 1995; Renner and Weibel 2011). Morphological studies of biofilms have revealed a wide variety of microscopic structures such as smooth, rough, fluffy, or filamentous and mushroom-like macrocolonies (Flemming and Wingender 2010; O'Toole et al. 2000). Mature bacterial biofilms are characterized by the production of large quantities of extracellular proteins, DNA, and polysaccharides that may play an important role in holding cells together and forming an encapsulated structure for isolating the

Fig. 12. *Xanthomonas citri* subsp. *citri* infection by phage Φ Xacm4-11 is type IV pili dependent. **A**, The 5- μ l aliquots of a 10^9 -fold dilution of a Φ Xacm4-11 stock solution were spotted on King's Broth soft agar (0.7% wt/vol) supplemented with *X. citri* subsp. *citri* strains at 1×10^8 CFU/ml and incubated at 28°C. The *X. citri* subsp. *citri* Δ *pilZ*_{XAC1133} knockout strain was complemented with a plasmid directing the expression of the wild-type *PilZ* protein (Δ *pilZc*) as well as the following *PilZ* mutants: *PilZ*_{Y25A}, *PilZ*_{F28A}, *PilZ*_{W69A}, *PilZ*_{E101A}, *PilZ*_{L104A}, and *PilZ* _{Δ 107-117}. Plaques indicative of bacterial lysis in a confluent culture background were observed 24 h postinoculation. **B**, Quantification of the *X. citri* subsp. *citri* lysis by the 2,3,5-triphenyltetrazolium chloride method. Black bars = *X. citri* subsp. *citri* strains treated with Φ Xacm4-11 and gray bars = untreated *X. citri* subsp. *citri* strains. Experiments were repeated three times, each with three replicates. Vertical bars represent the standard errors of the means.

bacterial community from the environment (Borlee et al. 2010; Flemming and Wingender 2010; O'Toole and Kolter 1998). In *P. aeruginosa*, T4P have been shown to promote bacterial adherence and aggregation as well as twitching motility that allows cells to move over each other to relocate to particular zones in the mature biofilm structure (Klausen et al. 2003; O'Toole and Kolter 1998). As with other bacteria, biofilm development in *X. citri* subsp. *citri* seems to be a complex and regulated process (Li and Wang 2011; Malamud et al. 2013; Zimaro et al. 2013). Malamud and associates (2013) observed that, at late stages of *X. citri* subsp. *citri* biofilm development, the mushroom-like structures open up and bacterial microcolonies detach and spread to form groups of pioneer cells that finally move to new environments. Our observations that T4P from *X. citri* subsp. *citri* are essential for both twitching motility and the development of columnar mushroom-like structures in a mature biofilm are consistent with this general framework and suggest that the T4P are important for dispersion of pioneer *X. citri* subsp. *citri* cells.

The role of T4P in phytopathogenic bacteria is not well understood and there seem to be significant differences in the plant pathogen models studied thus far. Piliated and nonpiliated *P. syringae* pv. *phaseolicola* inoculated into the host plant by infiltration caused halo blight but only the piliated strain caused symptoms when inoculated by the less invasive spraying method (Romantschuk and Bamford 1986). Similarly, the T4P from *P. syringae* pv. *tomato* DC3000 is implicated in bacterial fitness during foliar colonization and survival (Roine et al. 1998). *Acidovorax avenae* subsp. *citruilli* mutants in the T4P genes *pilA*, *pilM*, and *pilT* are affected in their ability to cause biofilm formation, twitching motility, and virulence in both early and later stages of the disease on melon seedlings (Bahar et al. 2009). Participation of T4P of the *Xanthomonas* genus in pathogenicity has been studied in only a few cases. No difference was observed in the growth of wild-type and $\Delta pilA$ mutant *X. campestris* pv. *vesicatoria* strains when inoculated by infiltrating into tomato leaves (Ojanen-Reuhs et al. 1997). A genome-wide mutational analysis showed that six genes that encode T4P components are important for the pathogenicity of *X. oryzae* pv. *oryzicola* on rice (Wang et al. 2007). In that study, disruption in the *pilM* gene that codes for a membrane protein involved in T4P assembly caused a severe reduction of virulence while more moderate effects were caused by the other five mutants (Wang et al. 2007). More recently, knockouts in the FimX and PilZ homologs of *X. oryzae* pv. *oryzae* resulted in reduced virulence on rice and reduced hypersensitive response induction in nonhost tobacco (Yang et al. 2014). The observed increase in transcription of the *pilA*_{XAC3241} gene during the early stages of host plant infection points to a possible role of T4P in the infection process by *X. citri* subsp. *citri*. For example, T4P-dependent twitching motility could be important during the stage of *X. citri* subsp. *citri* penetration into plant tissue and T4P-dependent biofilm structures could play a role in protection against antimicrobial agents released by the plant host. Finally, because *X. citri* subsp. *citri* has been shown to be able to survive outside of citrus tissues (Cubero et al. 2011; Pruvost et al. 2002), cooperative bacterial behaviors mediated by T4P could be expected to increase *X. citri* subsp. *citri* competitiveness and survival in antagonistic interactions with other bacterial species and eukaryotic microbes that it will encounter in various environmental niches.

MATERIALS AND METHODS

Bacterial strains, culture conditions, and media.

Escherichia coli DH5 α was used for DNA subcloning. Cells were cultivated at 37°C in LB medium. *X. citri* subsp. *citri*

were grown at 28°C in 2 $\times$ TY medium (tryptone at 16 g/liter, yeast extract at 10 g/liter, and NaCl at 5 g/liter, pH 7.0), SB medium (sucrose at 5 g/liter, yeast extract at 5 g/liter, peptone at 5 g/liter, and glutamic acid at 1 g/liter, pH 7.0) and KB medium (peptone at 20 g/liter, K₂HPO₄ at 1.5 g/liter, MgSO₄ • 7H₂O at 1.5 g/liter, and glycerol at 15 g/liter, pH 7.0). Antibiotics were used at the following concentrations: ampicillin at 100 μ g/ml, gentamycin at 20 μ g/ml, tetracycline at 20 μ g/ml, and kanamycin at 40 μ g/ml for *E. coli* and *X. citri* subsp. *citri*.

Construction of *X. citri* subsp. *citri* knockout strains.

In-frame deletions in the *pilZ*_{XAC1133}, *fimX*_{XAC2398}, *pilB*_{XAC3239}, and *fliC*_{XAC1975} genes by a two-step allelic exchange has been described previously (Guzzo et al. 2009). *pilA*_{XAC3241} and *pilT*_{XAC2924} genes were deleted by a two-step allelic exchange procedure. Briefly, approximately 1 kb corresponding to upstream and downstream regions of the genes were amplified by PCR (primers are listed in Supplementary Table S2). Each pair of fragments was ligated to produce a deleted version of the gene and then cloned into the pNPTS138 suicide vector (Alley Dickon, unpublished) in the restriction sites. These plasmids were introduced into *X. citri* subsp. *citri* by electroporation, and sucrose-sensitive and kanamycin- and ampicillin-resistant colonies were selected. Liquid cultures of these colonies were grown without selection; plated and individual colonies were selected for simultaneous kanamycin sensitivity and sucrose resistance. Deletions were confirmed by PCR. In order to complement the $\Delta pilA$ _{XAC3241}, $\Delta pilT$ _{XAC2924}, $\Delta pilZ$ _{XAC1133}, and $\Delta fimX$ _{XAC2398} knockouts, fragments coding for *pilA*_{XAC3241}, *pilT*_{XAC2924}, *pilZ*_{XAC1133}, and *fimX*_{XAC2398}, respectively were amplified and cloned into pUFR047, as described previously (Guzzo et al. 2009).

Recombinant PilA expression and antibody production.

The fragments corresponding to residues 37 to 151 of PilA_{XAC3240} (PilA_{XAC3240_37-151}) and the fragment coding for full-length PilA_{XAC3241} were cloned into the expression vectors pET28a and pET3a (Novagen), respectively, in the restriction sites. The proteins were expressed in *E. coli* strain BL21(DE3)STAR at 37°C. For purification of recombinant PilA_{XAC3240_37-151}, bacterial cells were resuspended 50 mM TrisHCl (pH 8.0) and 25% (wt/vol) saccharose and lysed by passage through a French Press, the insoluble fraction was resuspended in the same buffer containing 6 M urea, and the soluble fraction was applied to a His-Trap affinity column (GE Healthcare) previously equilibrated with 50 mM TrisHCl (pH 8.0), 300 mM NaCl, 20 mM imidazole, and 6 M urea. Bound proteins were eluted using a 0 to 1 M imidazole gradient over 20 column volumes. The fractions containing PilA_{XAC3240_37-151} were concentrated and applied to a Superdex 75 26/60 size-exclusion column (GE Healthcare) previously equilibrated with 50 mM TrisHCl (pH 8.0), 500 mM NaCl, 14 mM 2-mercaptoethanol, and 6 M urea. Purified PilA_{XAC3240_37-151} (200 μ g) was emulsified in complete Freud's adjuvant (Sigma-Aldrich) and used for the first immunization of a female New Zealand white rabbit. Three additional immunizations were performed every 7 days using a mixture with 200 μ g of PilA_{XAC3240_37-151} and incomplete Freud's adjuvant. Serum was collected and frozen at -80°C for future use.

PilA detection.

To detect pili on the surface of *X. citri* subsp. *citri* cells, wild-type and pilus knockout *X. citri* subsp. *citri* strains were plated in 15 large petri dishes on KB medium supplemented with 1% agar (wt/vol) and 2 mM CaCl₂ and incubated at 28°C for 3 days. Bacteria were collected with a plastic spatula and resuspended in 15 ml of phosphate buffer (pH 7.0). Surface

pili were mechanically sheared for 20 min in a blender. Bacteria and supernatant fractions were separated by centrifugation for 1 h at $16,000 \times g$. Proteins from the supernatant were precipitated with trichloroacetic acid, washed with acetone, and dissolved in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer. The bacterial pellet was also resuspended in sample buffer and boiled for 15 min. Protein samples were separated by SDS-PAGE followed by Western Blot analysis using polyclonal anti-PilA_{XAC3240} antibodies. These antibodies recognize both PilA_{XAC3240} and PilA_{XAC3241}.

RNA extraction and qRT-PCR.

For the quantitative analysis of *pilA*_{XAC3240}, *pilA*_{XAC3241}, and *xac3805* gene expression, the leaves of citrus plants were inoculated with *X. citri* subsp. *citri* by two different methods: infiltration of *X. citri* subsp. *citri* cells into plant tissue using a syringe without a needle or spraying *X. citri* subsp. *citri* cells onto the surface of leaves. Briefly, for each treatment, 36 leaves of three different orange plants were inoculated with *X. citri* subsp. *citri* at 1×10^7 CFU/ml. At different times postinfection, 12 leaves were collected and decontaminated with alcohol, and the midribs were removed. The leaves were cut into small pieces, sonicated for 20 min in sterile tubes containing 10 ml of distilled water, and maintained with shaking for 3 h at room temperature. Bacterial cells were collected by centrifugation and the bacterial pellet was washed with water and used for RNA isolation using Trizol reagent (Invitrogen), according to the manufacturer's instructions. Samples were treated with DNase I (RNase free; Thermo Scientific) to avoid DNA contamination during the cDNA synthesis. RNAs (1 μ g) were then subjected to reverse transcription using the Revert-Aid H Minus First-Strand cDNA Synthesis kit (Fermentas Life Science) using random hexamer primers, following the manufacturer's instructions. For analysis of *pilA*_{XAC3240}, *pilA*_{XAC3241}, and *xac3805* expression, quantitative real-time PCR was performed using a 7300 Real Time PCR System (Applied Biosystems). Reactions were performed with 200 ng of cDNA template and Maxima SYBR Green qPCR Master Mixes (Thermo Scientific) and 0.3 μ M gene-specific primers (Supplementary Table S3) under the following conditions: 95°C for 1 min followed by 40 cycles of 95°C for 15 s, 59°C for 20 s, and 72°C for 40 s. Specificity of the amplification reactions was assessed by agarose gel electrophoresis and melting curve analyses, which were run at 95°C for 15 s and 60°C for 15 s, followed by an increase in temperature from 60 to 85°C with continuous fluorescence recording. Relative expression was evaluated using the $2^{-\Delta\Delta CT}$ method normalizing relative to 16S *rRNA* (*xac4291*) and *gyrA* (*xac1631*) levels. All quantitative real-time PCR experiments were performed in triplicate.

Construction of GFP and CFP expression vectors.

The *GFP* gene coding for GFP was amplified from pMP2444 by PCR using the GFP1 and GFP2 oligonucleotides and the *CFP* gene coding for CFP was amplified from pECFP-C1 (Clontech Laboratories) by PCR using the CFP1 and CFP2 primers (Supplementary Table S4). The amplified products were cloned into the pBBRMCS-5 and pBBRMCS-2 plasmids kindly ceded by Professor Michael E. Kovach (Kovach et al. 1995), generating pBBR-5GFP, pBBR-2GFP, pBBR-5CFP, and pBBR-2CFP. These plasmids were used to transform specific *X. citri* subsp. *citri* strains, as indicated in the figure captions.

Subsurface twitching motility assays.

Twitching motility was assayed by the stab-inoculation method. *X. citri* subsp. *citri* strains were grown on LB agar (1.5% wt/vol) supplemented with the appropriate antibiotic at

28°C for 2 days. Using a sterile toothpick, *X. citri* subsp. *citri* cells were collected from an isolated colony and stabbed through SB agar (1% wt/vol) agar or through KB agar (1% wt/vol) supplemented with 2 mM CaCl₂ to the plastic surface of petri plates. After growth at 28°C for 5 days, the agar was removed and the zone of twitching motility between the agar and petri dish interface was visualized by staining with 0.1% (wt/vol) crystal violet (CV) at room temperature for 15 min, and unbound dye was removed by rinsing with distilled water. The subsurface twitching areas from *X. citri* subsp. *citri* were calculated by using the software Fiji (ImageJ). For microscopic analysis of subsurface twitching assays, *X. citri* subsp. *citri* strains were stabbed through KB agar (1%, wt/vol) supplemented with 2 mM CaCl₂ in a microscopy chamber covered with glass slides (Nu155411; Lab-Tek, NUNC). Chambers were statically incubated in a humidified chamber at 28°C for 2 days. Twitching motility was visualized by confocal laser-scanning microscopy (CLSM) (Zeiss LSM 510-Meta), where GFP was excited with a 30-mW argon laser at 488 nm and emission was collected using an LP505 emission filter. Alternatively, subsurface twitching motility of PilZ mutants and of the mixtures of wild-type and pilus knockout *X. citri* subsp. *citri* strains was analyzed using an inverted fluorescence microscope (Nikon Eclipse Ti-E) with specific excitation and emission filters for GFP and CFP. The images obtained were analyzed with Fiji (ImageJ) software. Three-dimensional images were reconstructed using Volume Viewer from Fiji (ImageJ) software.

Sliding motility assay.

Sliding motility was measured on the surface of SB medium plus 0.5% (wt/vol) agar. Molten medium was poured into plates and allowed to solidify and dry for 1 h. *X. citri* subsp. *citri* cells were grown in 2 $\times$ TY to mid-log phase at 28°C, diluted to absorbance at 600 nm of 0.3, and 5- μ l aliquots were used to inoculate the semisolid surface of the SB medium followed by incubation at 28°C for 2 to 4 days.

Biofilm formation assay.

For the analysis of biofilm formation, overnight cultures of the wild-type and pilus knockout *X. citri* subsp. *citri* strains expressing GFP in 2 $\times$ TY medium were collected by centrifugation, washed with fresh medium, and adjusted to an optical density at 600 nm (OD₆₀₀) of 0.5. Then, 4 μ l of this culture was diluted into 400 μ l of KB medium supplemented with 2% (wt/vol) glucose and pipetted into microscopy chambers (Nu155411; Lab-Tek, NUNC), covered with glass slides, and statically incubated at 28°C. Biofilm formation was visualized by CLSM (Zeiss LSM 510-Meta), where GFP was excited with a 30-mW argon laser at 488 nm and emission was measured through an LP505 filter (Zeiss). The images obtained were analyzed with Fiji (ImageJ) software. Three-dimensional images from bacterial biofilm were reconstructed using Volume Viewer from Fiji (ImageJ) software.

Adhesion assay.

To measure the *X. citri* subsp. *citri* ability to adhere to leaf surfaces, wild-type and T4P knockout *X. citri* subsp. *citri* strains were incubated overnight in 2 $\times$ TY at 28°C. Bacteria were recovered by centrifugation, washed, and resuspended in phosphate buffer (pH 7.0) at a concentration of 10^7 CFU/ml. Aliquots (20 μ l) of each bacterial suspension were spotted on the abaxial face of the orange plant's leaves and incubated for 6 h at 28°C in a humidified chamber. Leaves were washed with water to remove nonadherent cells, stained with a solution of 0.1% (wt/vol) CV at room temperature for 30 min, and then washed with water to remove unbound dye. Discs from the inoculated areas were ground and transferred to tubes with

95% (vol/vol) ethanol and the amount of bound dye was calculated by measuring absorbance at 570 nm. Experiments were performed in triplicate, applying 20 spots of each strain on leaf surfaces.

Bacteriophage plaque assay.

Overnight cultures *X. citri* subsp. *citri* wild-type and mutant strains were grown in 2xTY medium, collected by centrifugation, and resuspended in fresh medium at an OD₆₀₀ of 1. *X. citri* subsp. *citri* strains were mixed with warm liquefied KB agar (0.7% wt/vol) supplemented with 2 mM CaCl₂ to form the top agar layer overpoured into petri dishes carrying previously solidified KB agar (1% wt/vol) + 2 mM CaCl₂. Plates were dried for 10 min at room temperature and then spotted with 5 µl of dilutions (from 10³ to 10⁹) of the bacteriophage ΦXacm4-11 stock solution and incubated at 28°C for 24 h. For quantification of the bacterial lysis, overnight cultures of *X. citri* subsp. *citri* strains were adjusted to OD₆₀₀ of 0.01, and 100-µl aliquots were pipetted into the wells of sterile enzyme-linked immunosorbent assay microplates. Following incubation for 48 h at 28°C, to each well was added ΦXacm4-11 at 10⁸ PFU/ml and incubated for 24 h at 28°C. Then, 15 µl of 0.1% (wt/vol) 2,3,5-triphenyltetrazolium chloride was added to each well and incubated for 24 h at 28°C, after which the absorbance at 540 nm was measured.

EPS quantification.

Overnight cultures *X. citri* subsp. *citri* wild-type and mutant strains were grown in 2xTY medium, collected by centrifugation, washed in fresh medium, and resuspended at an OD₆₀₀ of 0.1 in SB medium with the addition of 2% (wt/vol) glucose and the appropriate antibiotic, and incubated for 72 h at 28°C with shaking. *X. citri* subsp. *citri* cultures were collected by centrifugation and EPS was precipitated by addition of two volumes of ethanol to the supernatants and incubated overnight at -20°C. Following filtration, the dry precipitate was weighed.

Plant inoculations and bacterial quantification.

Plant pathogenicity assays and bacterial growth curves of *X. citri* subsp. *citri* wild-type and mutant strains were performed as previously described (Dunger et al. 2005, 2007). *X. citri* subsp. *citri* strains, at concentration of 10⁷ CFU/ml in 10 mM MgCl₂, were inoculated into the abaxial side of orange leaves (*Citrus sinensis* cv. *lima*) using a needleless syringe (in plant growth) or spraying onto both side of orange leaves (epiphytic growth). Plants were maintained at 23 to 28°C, 58% relative humidity, with a photoperiod of 16 h. For in planta *X. citri* subsp. *citri* quantification, inoculated leaf discs were cut out at different timepoints after inoculation and macerated in 10 mM MgCl₂, and dilutions were plated on selective agar medium. For epiphytic bacterial quantification, inoculated leaf discs were cut out and placed into microcentrifuge tubes with 500 µl of water and sonicated for 20 min in a bath sonicator (Branson 1510), and dilutions were plated on selective agar medium.

ACKNOWLEDGMENTS

This work was supported by the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) to C. S. Farah (project number 2011/07777-5) and FAPESP fellowship to G. Dunger (project number 2011/22571-4). We thank F. Gueiros-Filho and B. Malnic (Institute of Chemistry, USP) for support and access to microscopy equipment, and G. Sgró for construction of the pBBR-MCS-CFP plasmid. G. Dunger and C. S. Farah conceived and designed the experiments; G. Dunger performed the experiments; G. Dunger, C. R. Guzzo, and C. S. Farah analyzed the data; G. Dunger, M. O. Andrade, C. R. Guzzo, J. B. Jones, and C. S. Farah contributed reagents and materials; G. Danger and C. S. Farah wrote the paper.

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