Type I and type IV pili of *Xylella fastidiosa* affect twitching motility, biofilm formation and cell–cell aggregation

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*Xylella fastidiosa*, an important phytopathogenic bacterium, causes serious plant diseases including Pierce’s disease of grapevine. It is reported here that type I and type IV pili of *X. fastidiosa* play different roles in twitching motility, biofilm formation and cell–cell aggregation. Type I pili are particularly important for biofilm formation and aggregation, whereas type IV pili are essential for motility, and also function in biofilm formation. Thirty twitching-defective mutants were generated with an EZ::TN transposome system, and several type-IV-pilus-associated genes were identified, including *fimT*, *pilX*, *pilY1*, *pilO* and *pilR*. Mutations in *fimT*, *pilX*, *pilO* or *pilR* resulted in a twitch-minus phenotype, whereas the *pilY1* mutant was twitching reduced. A mutation in *fimA* resulted in a biofilm-defective and twitching-enhanced phenotype. A *fimA/pilO* double mutant was twitch minus, and produced almost no visible biofilm. Transmission electron microscopy revealed that the pili, when present, were localized to one pole of the cell. Both type I and type IV pili were present in the wild-type isolate and the *pilY1* mutant, whereas only type I pili were present in the twitch-minus mutants. The *fimA* mutant produced no type I pili. The *fimA/pilO* double mutant produced neither type I nor type IV pili.

INTRODUCTION

*Xylella fastidiosa* is a Gram-negative non-flagellated bacterium that is limited to colonizing the water-conducting xylem vessels of host plants. A number of economically important diseases are caused by the bacterium, including Pierce’s disease of grapevine (*Vitis vinifera* L.), citrus variegated chlorosis, pear leaf scorch, and almond leaf scorch (Purcell, 1996, 1997). Disease symptoms are thought to occur as a result of water stress and nutritional deficiencies caused by blockage of the xylem vessels by the bacterium (Hopkins, 1989). The bacteria are transmitted to plants by xylem-feeding insect vectors, such as glassy-winged and blue-green sharpshooters (Hopkins, 1989; Tubajika et al., 2004; Hill & Purcell, 1995). To date, the molecular mechanisms of virulence of *X. fastidiosa*, as well as how it interacts with plant hosts, have not been elucidated. Hopkins (1989) reported that colonization and pathogenicity of the Pierce’s disease strains of *X. fastidiosa* are dependent on their ability to move within the xylem vessels, i.e. avirulent or weakly virulent strains multiply slowly, and rarely move from the point of inoculation. In support of this claim, it has recently been noted that some hypervirulent mutants migrate faster in plants than the wild-type isolate (Guilhabert & Kirkpatrick, 2005). The mechanism by which the bacteria are able to move from one location to another within the xylem is not entirely understood; however, we demonstrated recently that *X. fastidiosa* is able to migrate upstream, via twitch motility, using type IV pili (Meng et al., 2005), thus providing an explanation for downward migration in the plant; it was further shown that the bacterium migrates preferentially against a fluid current, suggesting that twitching motility provides the means for long-distance intra-plant movement. Type I pili are also present at the cell poles, and are thought to be involved in the development of biofilms and cell aggregation.

Twitching motility is a means of flagellar-independent bacterial movement over moist surfaces (Mattick, 2002). Twitching is known to function in host colonization, and it occurs through extension, attachment, and then retraction, of the polar type IV pili. It has been observed mostly in saprophytic bacteria and mammalian pathogens. Such motility in plant-associated bacteria has been observed in *Ralstonia solanacearum* (Kang et al., 2002; Liu et al., 2001) and *X. fastidiosa* (Meng et al., 2005). To date, approximately...
40 genes have been identified that are involved in the biogenesis and function of type IV pili in *Pseudomonas aeruginosa* (Mattick, 2002), including the genes that encode the major structural protein (PilA), and those that encode the minor proteins involved in formation of the base and/or tip of the pilus, e.g. PilE, PilV, PilW, PilX, PilY1, PilY2 and FimT. A number of other proteins are required for pilus assembly and retraction, e.g. PilB, PilC, PilD, PilF, PilM, PilN, PilO, PilP, PilQ, PilT and PilU. In addition, a set of genes encodes regulatory proteins that control the production of pili, and the activity of twitching motility in response to environmental stimuli; these genes include the two-component sensor–regulator pair pilS/pilR, and the genes associated with a chemosensory regulatory system: pilG– pilK and chpA–chpE. Studies have indicated that pilA mutants (pilin deficient) of *P. aeruginosa* are avirulent or have reduced virulence (Comolli et al., 1999; Hazlett et al., 1991), and that pilA, pilT and pilQ mutants of the phytopathogen *R. solanacearum* cause slower disease development, and less severe wilting symptoms, in tomato plants (Kang et al., 2002; Liu et al., 2001).

In addition to twitching motility, cell attachment and biofilm formation are thought to be important factors in pathogen virulence. Attachment to host surfaces by cell-surface adhesins is an essential early event in pathogenesis of many bacterial pathogens (Héléaine et al., 2005). Attachment is likely to function as the initial step in the formation of a biofilm, i.e. a community or population of micro-organisms attached to a solid surface that, as a structural unit, may provide survival advantages to the bacteria when exposed to different environments (de Souza et al., 2004). In *Escherichia coli*, for example, type I pili play a central role in cell attachment and biofilm formation (Blumer et al., 2005; Schilling et al., 2001). In other bacteria, type IV pili have also been shown to function in cell attachment and biofilm formation (Héléaine et al., 2005). For example, in *Neisseria meningitidis* and *Neisseria gonorrhoeae*, type IV pili facilitate bacterial attachment to human cells (Carbonnelle et al., 2005). Marques et al. (2002) demonstrated that *X. fastidiosa* isolates from various hosts formed biofilms on wood in an *in vitro* assay, and hypothesized that biofilm formation is likely to be a major virulence factor in disease caused by *X. fastidiosa*. Our recent study (Meng et al., 2005) revealed that the wild-type Temecula isolate of *X. fastidiosa* possesses both type I pili and type IV pili: type I pili play a central role in cell attachment and biofilm formation, and type IV pili mediate twitching motility on agar surfaces and against the flow of nutrient media in microfluidic chambers. A *fimA* mutant (type I pili deficient) was capable of moving upstream via twitching motility; however, pilB and pilQ mutants, which no longer produced type IV pili, did not exhibit a twitching phenotype. The pilB and pilQ mutants were also greatly impaired in their ability to migrate within grapevine shoots (Meng et al., 2005).

In this study, we report on the identity of several hitherto undescribed genes that are associated with the formation of type IV pili in *X. fastidiosa*. The association of specific genes with twitching motility, biofilm formation and cell–cell aggregation is determined.

**METHODS**

**Bacterial isolates and culture.** The wild-type isolate of *X. fastidiosa* Temecula (ATCC 700964), originally isolated from a grapevine with Pierce’s disease grown in Temecula, California (Van Sluys et al., 2003), was maintained on PW (Davis et al., 1981) agar modified by omitting phenol red, and by adding 3.5 g BSA l⁻¹ Fraction V solution (A-8918; Sigma) instead of 6.0 g BSA l⁻¹. Mutants were maintained on modified PW agar containing 50 mg kanamycin l⁻¹. Liquid modified PW was used for preparation of electrocompetent cells, and mutants were grown in PD2 (Davis et al., 1981) for DNA isolation and subsequent sequence analyses. *X. fastidiosa* isolates and mutants were stored at −80 °C in modified PW broth containing 7 % DMSO (final concentration; Sigma).

**Mutagenesis.** The EZ::TN Transposome system <Kan-2> kit (Epicentre) was used to generate *X. fastidiosa* mutants. Electrocompetent cells were prepared as described by Guilhabert et al. (2001). Electroporation was conducted at 2500 V, 200 Ω and 25 μF for 5 ms, and the electrocompetent cells were plated on modified PW agar plus kanamycin. A second mutation in the *fimA* mutant 6E11 (Meng et al., 2005) was accomplished with the EZ::TN Transposome <DHFR-1> kit (Epicentre). The electrocompetent cells were subsequently plated on modified PW agar plus kanamycin (50 mg l⁻¹) and trimethoprim (250 mg l⁻¹). Individual colonies of putative mutants were verified by PCR for Tn5 insertions.

**Screening for twitching motility mutants.** Mutants were screened for twitching motility on modified PW containing 1.8 g BSA l⁻¹, and solidified with 1.2 % agar. Bacteria were picked from modified PW agar with a sterile toothpick, and gently spotted onto PW agar surfaces. The plates were incubated at 28 °C for 3 days, after which the edge morphology of the colonies was examined using a dissecting microscope (SZX12; Olympus). Colonies with a peripheral fringe were designated as twitch positive (Meng et al., 2005). Colonies lacking a peripheral fringe were checked several times to verify the twitch-minus phenotype. Some mutants were further examined microscopically in microfabricated fluidic chambers using time-lapse imaging (Meng et al., 2005).

**Sequence analysis.** Genomic DNA from twitching-defective mutants was digested with EcoRI, generating fragments that included the Tn5 insertion. The fragments were then cloned into the EcoRI site of pUC18. DNA sequences of disrupted genes were determined by using transposon-specific primers (provided by Epicentre) reading sequences outwards from the transposon. About 800–1000 nt were sequenced for each mutant of interest. The genomic locations of Tn5 insertions were identified using a BLAST search of the *X. fastidiosa* Temecula genome database (http://aeg.lib.cic.unicamp.br/world/xfpd/).

**Southern blotting.** Confirmation of the presence of a single Tn5 insertion in mutants of interest was performed by Southern analysis. Genomic DNA isolated from twitching-defective mutants was digested with *Hind*III, and electrophoretically separated on 1 % agarose. DNAs were alkali denatured, transferred to a hybridization transfer membrane (NEM Life Science Products), and fixed by baking at 85–90 °C for 2 h. Tn5 DNA was PCR amplified, purified, and labelled with DIG High Prime DNA Labeling and Detection Starter kit 1 (Roche). The labelled Tn5 was used as a hybridization probe in Southern blot analysis of genomic DNAs from the mutants.

**Electron microscopy.** Cells were generally obtained from the periphery of 2-day-old colonies. Cells were mixed with distilled water,
deposited on Formvar-coated grids, and subsequently stained with either phosphotungstic acid or uranyl acetate, and examined with a JEOL S-100 transmission electron microscope.

**Biofilm formation and cell–cell aggregation.** Cells taken from 5- to 7-day-old cultures grown on PW agar plates were suspended in liquid PD2, and adjusted to an OD$_{600}$ of 0.1. A 100 µl cell suspension was added to 1 ml PD2 in polystyrene tubes, polypropylene tubes, and glass tubes, and grown at 28 °C for 7 days, without agitation. Subsequently, the medium was stained with 100 µl 0.1 % crystal violet for 20 min, and rinsed three times with distilled water. The presence of a biofilm was visualized as a purple ring on the tube side wall, usually at the air–medium interface (O'Toole et al., 1999).

In a separate study, biofilm development was assessed on the inner surface of 250 ml glass Erlenmeyer flasks, along with aggregation of *X. fastidiosa* cells at the bottom of the medium in the same flasks. For these observations, 300 µl cell suspension, as described above, was added to 50 ml PD2. Flasks were incubated at room temperature (approximately 22 °C), with agitation (195 r.p.m.), for 7–10 days. Following assessment of biofilm development and cell aggregation, the contents of the flasks were removed, and the integrity of the side wall biofilm was determined by adding 50 ml distilled water, and swirling vigorously to disperse cells that were not tightly bound to the flasks.

**Growth rates.** Cells from 5- to 7-day-old cultures grown on modified PW agar were suspended in modified PW broth (3.5 g BSA l$^{-1}$), and adjusted to an OD$_{600}$ of 0.1. A 300 µl cell suspension was added to 10 ml PW broth in glass culture tubes (18 cm long), and incubated at 28 °C, with continuous agitation at 195 r.p.m. Cell density was determined at regular intervals by measuring OD$_{600}$. The experiment was repeated twice, independently.

**RESULTS**

**Mutagenesis and screening of twitching mutants**

High transformation efficiency was achieved (up to $7.5 \times 10^8$ transformants per µg transposon DNA) when Tn5 transposomes were electroporated into *X. fastidiosa*. Transposon insertion (fragment size 700–800 bp) was detected in all mutants tested by PCR, but was not present in the wild-type *X. fastidiosa* isolate. Putative twitch-minus mutants were selected by the presence of a smooth colony margin, i.e. the absence of a twitching-associated peripheral fringe (Fig. 1) (Meng et al., 2005). Thirty Twitching-defective mutants were obtained from approximately 3000 KanR insertion mutants, seven of which are described in this report (Table 1). Southern analysis confirmed that a single insertion occurred in the genome of each mutant selected for further study (data not shown).

In addition to single insertion mutants, a second round of mutagenesis into the previously described mutant 6E11, which was deficient for type I pili, but retained type IV pili and a twitching phenotype (Meng et al., 2005), was performed to create a ‘double’ mutant that lacked both type I and type IV pili. Six non-twitching double mutants were obtained, one of which (DM12) is reported herein.

**Sequence analysis of mutants**

The DNA sequence of the regions flanking the transposon insertions of the twitching mutants revealed that in the 30 mutants the transposon had inserted into 12 different ORFs. Those in which insertions occurred in ORFs that are homologous of pilus-related genes in *P. aeruginosa*, including PD0019 (*fimT*, one mutant), PD0022 (*pilX*, one mutant), PD0023 (*pilY1*, two mutants), PD1693 (*pilO*, two mutants), and PD1928 (*pilR*, two mutants), were selected for further investigation. In these organisms, the disrupted genes reside in four different pilus-related gene clusters (Fig. 2). We have previously reported mutations in *pilB* and *pilQ* (Fig. 2; Meng et al., 2005). DNA sequence alignment analysis indicated high levels of identity between these genes compared with type-IV-pilus-associated genes in *P. aeruginosa* (Table 1). In the previously described mutant 6E11 (Meng et al., 2005), the transposon insertion occurred in ORF PD0062 (Fig. 2), which corresponded to the fimA gene of *E. coli* whose product is the major protein subunit precursor for type I pili. The second insertions in mutant 6E11
occurred in PD1923 (pilC in DM11 and DM15), PD1693 (pilO in DM12), PD1671 (DM13), PD0609 (DM14) and PD0022 (pilX in DM16), respectively. Among these double mutants, DM12 (fimA/pilO) (Fig. 2) was selected for further study because it possessed neither type I nor type IV pili. The pilO gene resides in operon pilMNOPQ (Van Sluys et al., 2003), whose homologues in P. aeruginosa are required for type IV pilus assembly (Martin et al., 1995; Mattick, 2002).

### Pili

As previously reported, *X. fastidiosa* possesses both type I pili (0.4–1.0 µm in length) and type IV pili (1.0–5.8 µm in length) (Fig. 3a; Meng et al., 2005). Transmission electron microscopy revealed that the twitch-minus mutants TM1 (pilO), TM7 (pilR), TM13 (fimT) and 20D10 (pilX) lacked type IV pili, but possessed type I pili (Fig. 3b). The fimA mutant 6E11 (Fig. 3d) was used to create the double mutant DM12, which lacked both types of pili (Fig. 3c). Both type I and type IV pili were observed in the pilY1 mutant TM14 (not shown), and they appeared similar to those observed in the wild-type isolate.

### Twitching motility of wild-type and mutants

Mutants with peripheral fringe morphologies of colonies that differed from wild-type *X. fastidiosa* were selected (Fig. 1). Mutants TM1 (pilO), TM7 (pilR), TM13 (fimT) and 20D10 (pilX) exhibited smooth colony margins on both

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### Table 1. Mutants and identities of the disrupted genes in *X. fastidiosa* with their homologues in *P. aeruginosa*

<table>
<thead>
<tr>
<th>X. fastidiosa</th>
<th>P. aeruginosa</th>
<th>Percentage identity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mutant</td>
<td>Gene (ORF)</td>
<td>Predicted gene product</td>
</tr>
<tr>
<td>TM13*</td>
<td>fimT (PD0019)</td>
<td>fimT (PA4549) Type IV fimbrial biogenesis protein FimT 29</td>
</tr>
<tr>
<td>20D10*</td>
<td>pilX (PD0022)</td>
<td>pilX (PA4553) Type IV fimbrial biogenesis protein PilX 24</td>
</tr>
<tr>
<td>TM14†</td>
<td>pilY1 (PD0023)</td>
<td>pilY1 (PA4554) Type IV fimbrial biogenesis protein PilY1 31</td>
</tr>
<tr>
<td>5A7*</td>
<td>pilQ (PD1691)</td>
<td>pilQ (PA5040) Type IV fimbrial biogenesis outer-membrane protein. PilQ precursor 39</td>
</tr>
<tr>
<td>TM1*</td>
<td>pilO (PD1693)</td>
<td>pilO (PA5042) Type IV fimbrial biogenesis protein PilO 41</td>
</tr>
<tr>
<td>1A2*</td>
<td>pilB (PD1927)</td>
<td>pilB (PA4526) Type IV fimbrial biogenesis protein PilB 56</td>
</tr>
<tr>
<td>TM7*</td>
<td>pilR (PD1928)</td>
<td>pilR (PA4547) Two-component response regulator PilR 58</td>
</tr>
</tbody>
</table>

*Colony margin smooth and not fringed.
†Colony margin smooth and not fringed, occasionally crenulated.
§Identity to *P. aeruginosa*.
PD2 (data not shown) and modified PW agar surfaces, indicating that they lacked twitching motility (Fig. 1), and were similar to the phenotype previously reported in \textit{pilB} and \textit{pilQ} mutants (Meng \textit{et al.}, 2005). Mutant TM14 (\textit{pilY1}) exhibited a crenulated colony margin, suggesting reduced, but not completely impaired, twitching motility. Subsequently, time-lapse examination of this mutant showed that it retained partial motility (a video of this is available as supplementary data with the online version of this paper, and also at www.nysaes.cornell.edu/pp/faculty/hoch/movies). Mutant 6E11 (\textit{fimA}), previously reported to be twitch positive (Meng \textit{et al.}, 2005; also see http://www.nysaes.cornell.edu/pp/faculty/hoch/movies), exhibited a peripheral colony fringe similar to that observed in the wild-type. Further observation in this study revealed that the peripheral fringe of 6Ell colonies was nearly always wider than the fringe of the wild-type isolate, suggesting a greater rate of motility in 6E11 (Fig. 1). The double mutant DM12 (\textit{fimA/pilO}) did not exhibit a colony peripheral fringe, was incapable of twitching, and lacked both type I and type IV pili (Fig. 1).

**Biofilms, cell aggregates, and growth rates**

We have previously demonstrated that biofilm formation in \textit{X. fastidiosa} is influenced by the presence of type I and type IV pili (Meng \textit{et al.}, 2005). In this study we further characterized the development of biofilms and cell–cell aggregates in newly generated pilus mutants on different surfaces and media. Since it has been observed that cells of \textit{X. fastidiosa} Temecula attach poorly to surfaces and to each other when cultured in PW medium, PD2 was used for observations of biofilm formation. When \textit{X. fastidiosa} wild-type and twitching-defective mutants were cultured without agitation, they formed visible biofilms on polystyrene and polypolypropylene surfaces; in contrast, 6E11 and DM12 exhibited significantly reduced biofilms on similar surfaces (Fig. 4). The twitch-minus mutants that lacked only type IV pili formed more robust biofilms on polystyrene and glass surfaces than the wild-type isolate; however, no differences were observed on polypolypropylene with these same mutants, suggesting that the surface material greatly affects attachment of the bacteria. We also tested biofilm formation by the wild-type, and TM1, 6E11 and DM12, cultured in glass Erlenmeyer flasks with continuous agitation (Fig. 5a). TM1 formed significantly more biofilm than did the wild-type isolate. This result was similar to that reported for the \textit{pilB} and \textit{pilQ} mutants (Meng \textit{et al.}, 2005). The biofilm formed by 6E11 was visibly reduced as compared to the wild-type isolate or TM1 (Fig. 5a). DM12 developed no visible biofilm on the flasks (Fig. 5a). Furthermore, it was noted that the biofilms formed by the wild-type and 6E11 were easily removed from the flask surfaces by swirling in distilled water, whereas the biofilm formed by TM1 remained intact (data not shown).

The quantity of non-attached cell aggregates at the bottom of the culture vessels was approximately inversely proportional to the amount of biofilm on the flask side walls (Fig. 5). In addition, there were distinct differences in the size and morphologies of these aggregates. DM12 aggregates were not only numerous but small in size and lens-shaped.
compared with the other mutants and the wild-type (Fig. 5). The TM1 mutant consistently produced the largest aggregates, whereas the wild-type and the 6E11 mutant, both with the longer type IV pili, produced loosely compact aggregates (Fig. 5). These results suggest that afimbrial adhesions play an important role in cell–cell aggregation. In contrast, the aggregates formed by the wild-type, TM1 and 6E11 were larger than those formed by DM12, suggesting that the presence of pili causes more cells to clump together, and form larger aggregates. Growth rates of the various mutants did not differ significantly from that of wild-type X. fastidiosa (not shown); therefore, the twitching phenotypes observed in the mutants were not the result of altered growth ability.

**DISCUSSION**

The genome of the Pierce's disease strain of X. fastidiosa contains at least 25 putative pil genes that are likely to be associated with type IV pilus formation and twitching motility (Van Sluys et al., 2003; Meng et al., 2005). The mutants described in the current study are associated with genes that are homologous to the pil genes in P. aeruginosa. In P. aeruginosa, FimT, PilX and PilY1 are minor proteins that are predicted to be associated with the base and/or tip of the type IV pilus (Mattick, 2002). In N. meningitidis, PilX has been reported to be essential for bacterial aggregation and pilus-mediated attachment to human cells (Hélaine et al., 2005). PilY1 in P. aeruginosa is associated with the cell membrane, and is required for the development of extra-cellular pili and twitching (Alm et al., 1996). PilY1 is a homologue of PilC2, which is a pilus tip adhesion protein in N. gonorrhoeae (Alm et al., 1996). We have observed type IV pili in the pilY1 mutant; this is to be expected if the gene encodes the tip adhesion protein. The mutation would presumably affect the ability of the pilus to attach to the agar surface, thereby affecting twitching motility. The pilY1 mutant displayed a crenulated colony periphery, and was shown to twitch less effectively than the wild-type.

The pilO gene is located in a putative operon consisting of PD1691–PD1695 (pilMNOPQ). The predicted pilO gene product shares 41% identity with PilO of P. aeruginosa (Table 1). In P. aeruginosa, this operon is required for pilus assembly (Martin et al., 1995). A pilQ mutant in X. fastidiosa failed to produce type IV pili, and had a twitch-minus phenotype (Meng et al., 2005). Another pil gene cluster, PD1922–PD1928, contains pilB and pilR, whose predicted protein products share 56 and 58% identities, respectively, with the homologous PilB and PilR in P. aeruginosa (Table 1). The gene cluster in P. aeruginosa is thought to function in type IV pilus biogenesis and twitching motility (Mattick, 2002; Hobbs et al., 1993). PilS and PilR belong to the family of two-component transcriptional regulatory systems that have been described in many bacterial species (Hobbs et al., 1993). PilS is a predicted sensory protein that, when stimulated by the appropriate environmental signals, activates PilR through kinase activity; PilR then regulates transcription of pilA (Hobbs et al., 1993). We have shown that the pilR mutant lacks type IV pili, and does not twitch, which may result from lack of pilA expression.

An interesting characteristic of X. fastidiosa is that it possesses both type I and type IV pili, predominantly at one cell pole. The fimA gene is located in the cluster PD0058–PD0062 (Van Sluys et al., 2003). Homologues of this gene cluster in E. coli are required for biofilm formation, and FimA is the major subunit for type I pili (Blumer et al., 2005; Schilling et al., 2001). The predicted protein product of fimA in X. fastidiosa shares 23.1% identity with the homologue FimA in E. coli K-12. The fimA mutant of X. fastidiosa was unable to produce type I pili, but was able to produce type IV pili, and maintain twitching motility. Feil et al. (2003) disrupted fimA and fimH in X. fastidiosa, and observed that fimB (i.e. pilus) size and number, as well as cell aggregation and aggregate size, were reduced in both fimA and fimH mutants. Interestingly, the mutants remained pathogenic in grapevines.

**Pili and twitching motility**

Type-IV-pilus-mediated twitching motility has been documented for many bacteria (Hobbs et al., 1993; Liu et al., 2001; Meng et al., 2005). Without type IV pili, X. fastidiosa cells were unable to twitch, whereas the fimA mutant remained twitch positive. We observed, based on colony peripheral fringe width, that the twitching motility of the fimA mutant (6E11) was enhanced compared with the wild-type; this provides further evidence that type I pili are not required for twitching motility. The enhanced twitching motility of the fimA mutant suggests that the presence of type I pili may partially restrict cell movement.

**Biofilm formation and cell–cell aggregation**

Type I pili of X. fastidiosa play fundamental roles in biofilm formation and cell–cell aggregation. The twitch-minus mutants TM1, TM7, TM13 and 20D10, which lack type IV pili, produced more abundant biofilms and larger cell aggregates than the wild-type isolate or any of the other mutants (Figs 4 and 5). Mutants that possessed only type IV pili (6E11) exhibited less pronounced biofilms, and the mutant with no pili (DM12) did not produce an easily discernible biofilm. For DM12, afimbrial cell surface adhesins may have led to the formation of the numerous small aggregates. This result is consistent with a report (Guilhabert & Kirkpatrick, 2005) that haemagglutinins mediate cell–cell aggregation in X. fastidiosa. When the gene lxa (PD2118) was disrupted, the ability to form aggregates in liquid culture was impaired. The haemagglutinin adhesions produced by Erwinia chrysanthemi have also been shown to contribute to aggregation (Rojas et al., 2002). When either or both type I and type IV pili are present, larger aggregates are formed, suggesting a role for pili (Figs 4 and 5). It is not surprising that some biofilm formation occurred in the mutant with only type IV pili (6E11), since these pili are involved in twitching motility, which is a
mechanism that requires cell attachment to substrata. Similar roles of attachment for type IV pili have been shown in *P. aeruginosa* and other bacteria (O’Toole & Kolter, 1998; Scheuerpflug et al., 1999). In addition, the ability to attach to host surfaces has been shown to be essential for virulence in other systems (Merz & So, 2000; Mattick, 2002). We show that type IV pili also play an important role in surface attachment and biofilm formation. Our results suggest that type IV pili play a role in biofilm formation; however, the biofilm density seems to be greatly influenced by the presence of type I pili.

The results of this study improve our understanding of how *X. fastidiosa* pili influence twitching motility, biofilm formation and cell–cell aggregation. The roles of the identified pilus genes in movement and colonization within plants, and ultimately disease, will be further investigated. The elucidation of genetic mechanisms associated with virulence of *X. fastidiosa* may be exploited for the development of novel controls for Pierce’s disease.

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