

THE ROLES THAT DIFFERENT PILI CLASSES IN *XYLELLA FASTIDIOSA* PLAY IN COLONIZATION OF GRAPEVINES AND PIERCE'S DISEASE PATHOGENESIS

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ABSTRACT

Type I and type IV pili of *Xylella fastidiosa* play different roles in twitching motility, biofilm formation, and cell-cell aggregation. Thirty twitching mutants were generated with an EZ::TN transposome system and type IV pilus-associated genes were identified, including *fimT*, *pilX*, *pilY1*, *pilO*, and *pilR*. Mutations in all resulted in a twitch-minus phenotype except that *pilY1* mutant was twitching-reduced. A *fimA* mutant lacked type I pili and altered biofilm development and twitching. A *fimA/pilO* double mutant lacked both classes of pili, was twitch-minus and produced almost no visible biofilm. The gene for the type IV pilin (*pilA*), was cloned and expressed (predicted 15 KDa protein). The pilin sequence is 38% and 55% identical to that of the type IV pilin from *Pseudomonas aeruginosa* and *P. syringae* pv. tomato, respectively. A monoclonal antibody against the *pilA* gene product (prepilin) is being prepared.

INTRODUCTION

Xylella fastidiosa (*Xf*) has both type I pili and type IV pili located at one pole of the cells, and exhibits twitching motility and biofilm formation (Meng et al., 2005). Twitching functions in host colonization of many gram-negative bacteria. Approximately 40 genes have been identified that are involved in the biogenesis and function of type IV pili in *P. aeruginosa* (Mattick, 2002), including those encoding structural and regulatory proteins. In several bacteria, type IV pili are known to function in attachment and biofilm formation (Hélaine et al., 2005, Schilling et al., 2001); known virulence factors. The main structural protein of IV pili, pilin, is encoded by *pilA* and is essential for the twitching motility in *P. aeruginosa* and *P. stutzeri* (Mattick et al., 2002; Graupner et al., 2000).

Our recent study revealed that type I pili play a central role in cell attachment and biofilm formation, and that type IV pili mediate twitching motility against a flowing current in microfluidic chambers. A *fimA* mutant (no type I pili) was capable of twitching motility. In contrast *pilB* and *pilQ* mutants (no type IV pili) did not twitch and were greatly impaired in their ability to migrate downward in grapevine shoots (Meng et al., 2005). We have identified several previously undescribed genes in *Xf* that are associated with pili development and their associated phenotypes. We have cloned and expressed *pilA* and its product, pilin, the primary structural protein of type IV pili. Antibodies to PilA and other surface proteins will be used for development of diagnostic tests and eventually in the development of novel controls for Pierce's Disease.

OBJECTIVES

1. Characterize the putative type I pili gene cluster and phenotypes associated with genes.
2. Characterize two additional gene clusters that are likely to be involved in regulation of type IV pili and related functions.
3. Development of monoclonal antibodies to *Xf*.

RESULTS

Characterization of gene clusters associated with pil genes. Thirty twitching-defective mutants, representing 12 different open reading frames, were obtained from approximately 3000 Kan^R insertion mutants generated via the EZ::TN <Kan-2> system. Insertions occurred in homologs of pilus-related genes of *P. aeruginosa*, including PD0019 (*fimT*), PD0022 (*pilX*), PD0023 (*pilY1*), PD1693 (*pilO*), and PD1928 (*pilR*) that reside in four different gene clusters (Table 1; Figure 1). Open reading frame PD0062 corresponds to the *fimA* gene of *E. coli* (precursor for type I pili). A second round of mutagenesis in mutant 6E11 (lacks type I pili) was performed with EZ::TN <DHFR-1> system to select mutants that lacked both pilus types. Six non-twitching mutants were obtained having insertions in PD1923 (*pilC* in DM11, DM15), PD1693 (*pilO* in DM12), PD1671 (DM13), PD0609 (DM14) and PD0022 (*pilX* in DM16), respectively. DM12 (*fimA/pilO*) was selected for further study. *pilO* resides in operon *pilMNOPQ* (Van Sluys et al., 2003). Homologs in *P. aeruginosa* are required for type IV pilus assembly (Mattick, 2002).

Table 1.

<i>Xylella fastidiosa</i>		<i>Pseudomonas aeruginosa</i>		Percent Identity ‡
Mutant	Gene (ORF)	Gene (ORF)	Predicted Gene Product	
TM13 *	<i>fimT</i> (PD0019)	<i>fimT</i> (PA4549)	Type IV fimbrial biogenesis protein FimT	29
20D10 *	<i>pilX</i> (PD0022)	<i>pilX</i> (PA4553)	Type IV fimbrial biogenesis protein PilX	24
TM14 †	<i>pilY1</i> (PD0023)	<i>pilY1</i> (PA4554)	Type IV fimbrial biogenesis protein PilY1	31
5A7 *	<i>pilQ</i> (PD1691)	<i>pilQ</i> (PA5040)	Type IV fimbrial biogenesis outer membrane protein. PilQ precursor	39
TM1 *	<i>pilO</i> (PD1693)	<i>pilO</i> (PA5042)	Type IV fimbrial biogenesis protein PilO	41
1A2 *	<i>pilB</i> (PD1927)	<i>pilB</i> (PA4526)	Type IV fimbrial biogenesis protein PilB	56
TM7 *	<i>pilR</i> (PD1928)	<i>pilR</i> (PA4547)	Two-component response regulator PilR	58

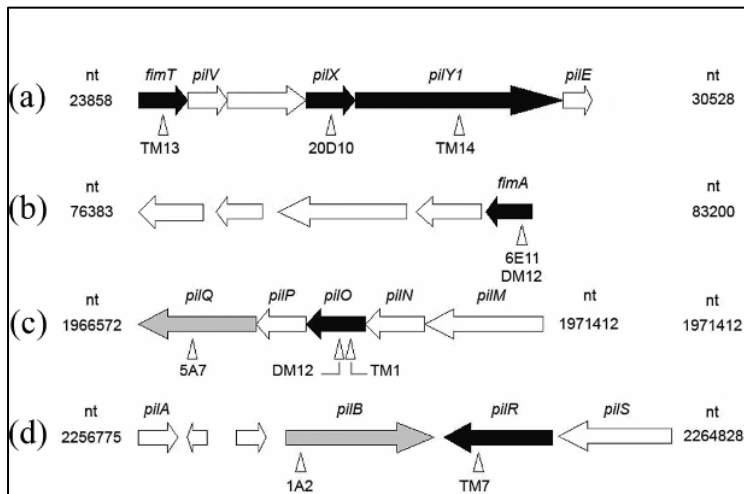


Figure 1.

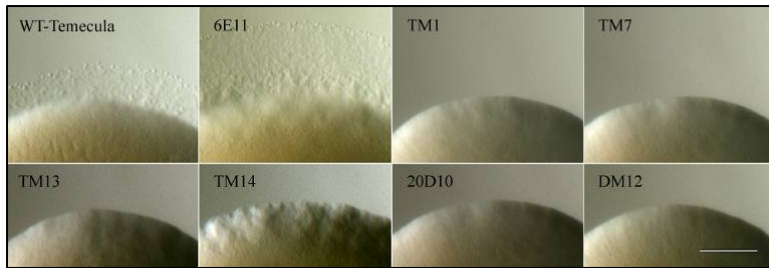


Figure 2.

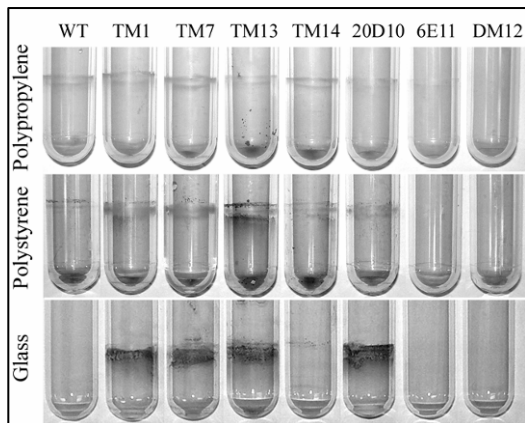


Figure 3.

TEM revealed that mutants TM1 (*pilO*), TM7 (*pilR*), TM13 (*fimT*), and 20D10 (*pilX*) lacked type IV pili, but still possessed type I pili. The double mutant DM12, lacked both classes of pili. Type I and type IV pili were both present in the *pilY1* mutant, TM14.

Twitching motility of wild-type and mutants.

Mutants TM1 (*pilO*), TM7 (*pilR*), TM13 (*fimT*), and 20D10 (*pilX*) exhibited smooth colony margins on modified PW agar surfaces, indicating that they lacked twitching motility as shown in Figure 2.

Mutant TM14 (*pilY1*) exhibited a crenulated colony margin, suggesting reduced twitching motility. The peripheral fringe of 6E11 colonies was nearly always wider than the fringe of the wild-type isolate, suggesting enhanced motility.

This infers that the presence of type I pili may restrict motility, possibly by enhancing attachment and aggregation of cells. The double mutant DM12 (*fimA pilO*) exhibited no fringe or twitching.

Biofilms, cell aggregates, and growth rates.

Wild-type and twitching-defective mutants formed biofilms on polystyrene and polypropylene surfaces; 6E11 and DM12 exhibited significantly reduced biofilms (Figure.

3). Mutants lacking only type IV pili formed more robust biofilms on polystyrene and glass than the wild-type; however, no differences were observed on polypropylene suggesting that the surface material greatly impacts attachment of the bacteria. Biofilm formation by wild-type, TM1, 6E11, and DM12 was tested in Erlenmeyer flasks with continuous agitation (Figure 4a). TM1 formed significantly more biofilm than did the wild-type isolate. The biofilm formed by 6E11 was visibly reduced as compared to the wild-type isolate or TM1 and DM12 developed no visible biofilm (Figure 4a). 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). Thus the presence of pili make up also affects biofilm integrity.

The quantity of non-attached cell aggregates at the bottom of the culture vessels was proportional to the amount of biofilm on the flask side walls (Figure 4b) and distinct differences in the size and morphologies of aggregates was apparent. DM12 aggregates were

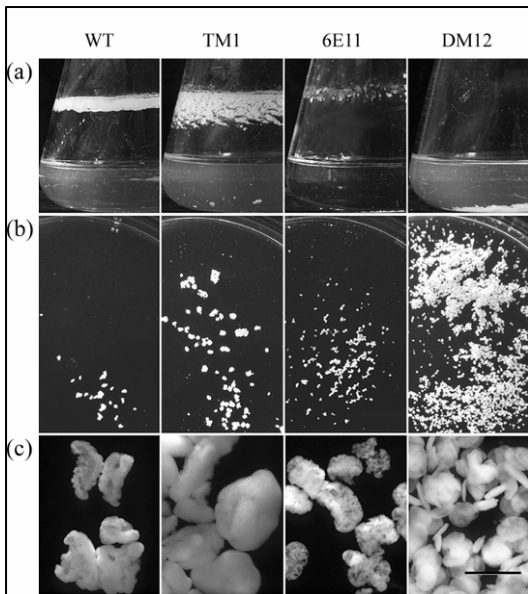


Figure 4.

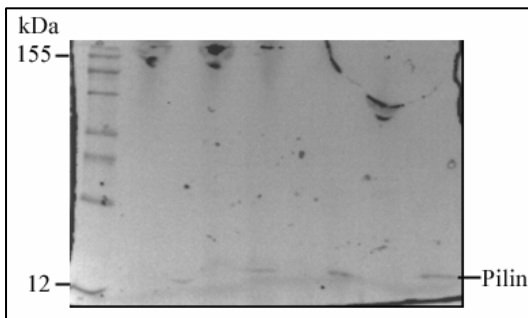


Figure 5.

numerous, small and lens-shaped compared to the other mutants and the wild-type (Figure 4c). TM1 consistently produced the largest aggregates, whereas wild-type and the 6E11 mutant produced loosely compact aggregates suggesting that afimbrial adhesions play a role in cell-cell aggregation. Aggregates of TM or 6E11 were larger than DM12, suggesting that pili affect the formation of larger aggregates. Growth rates of the various mutants did not differ significantly from wild-type (data not shown).

Cloning and expression of Type IV pilin. The gene for the type IV pilin (*pilA* gene product) from *X. fastidiosa*, was cloned and expressed in *E. coli* BL21 (DE3) cells. The pilin (deduced 15 KDa) sequence is 38% and 55% identical to those of the type IV pilin from *Pseudomonas aeruginosa* PAO1 and *P. syringae* pv. tomato, respectively (Figure 5). A monoclonal antibody against *pilA* encoded pilin, using phage display technology, is being prepared.

CONCLUSIONS

Our results show that genes *pilO*, *pilR*, *fimT*, *pilX* are required for type IV pili formation and twitching motility in *Xylella fastidiosa*, and *pilY1* affects twitching to a lesser degree. Twitching appears to be important in plant colonization by the pathogen. The type IV pili also are involved in biofilm formation and cell-cell aggregation and thus may play a role in virulence. Type I pili play a central role in biofilm formation and cell-cell aggregation. Cloning and expression of Type IV pilin have implications for understanding Type IV pili function and provide a useful background for the further characterization of the precise function of pilin protein in this process.

REFERENCES

- Feil, H., W. S. Feil, J. C. Detter, A. H. Purcell, & S. E. Lindow. (2003). Site-directed disruption of the *fimA* and *fimF* genes of *Xylella fastidiosa*. *Phytopathology* 93:675-682.
- Graupner, S., V. Frey, R. Hashemi, et al. (2000). Type IV Pilus Genes *pilA* and *pilC* of *Pseudomonas stutzeri* Are Required for Natural Genetic Transformation, and *pilA* Can Be Replaced by Corresponding Genes from Nontransformable Species. *J Bacteriology* 182, 2184-2190.
- Guilhabert, M. R. & B. C. Kirkpatrick. (2005). Identification of *Xylella fastidiosa* antivirulence genes: Hemagglutinin adhesins contribute to *X. fastidiosa* biofilm maturation and colonization and attenuate virulence. *MPMI* 18, 856-868.
- Mattick, J. S. (2002). Type IV pili and twitching motility. *Annu Rev Microbiol* 56, 289-314.
- Meng, Y., Y. Li, C. D. Galvani, G. Hao, J. N. Turner, T. J. Burr, & H. C. Hoch. (2005). Upstream migration of *Xylella fastidiosa* via pilus-driven twitching motility. *J Bacteriol* 187, 5560-5567.
- Schilling, J. D., M. A. Mulvey & S. J. Hultgren. (2001). Structure and function of *Escherichia coli* type I pili: New insight into the pathogenesis of urinary tract infections. *J Infectious Diseases* 183, S36-40.
- Simpson, A. J., F. C. Reinach, P. Arruda, et al. (2000). The genome sequence of the plant pathogen *Xylella fastidiosa*. *Nature*, 406: 151-157.
- Tubajika, K. M., E. L. Civerolo, M. A. Ciomperlik, D. A. Luvisi & J. M. Hashim. (2004). Analysis of the spatial patterns of Pierce's disease incidence in the lower San Joaquin Valley in California. *Phytopathology* 94, 1136-1144.
- Van Sluys M. A., M. C. de Oliveira, C. B. Monteiro-Vitorello, et al. (2003). Comparative analyses of the complete genome sequences of Pierce's disease and citrus variegated chlorosis strains of *Xylella fastidiosa*. *J Bacteriology*, 185, 1018-1026.

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TOWARDS THE DEVELOPMENT OF A SEQUENCE-BASED PCR SYSTEM FOR DETECTION AND GENOMIC STUDY OF *XYLELLA FASTIDIOSA* STRAINS IMPORTANT TO CALIFORNIA

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ABSTRACT

We report two of our recent efforts for improvement of PCR detection and genomic analysis of *Xylella fastidiosa* (*Xf*). We evaluated the use of PCR primers from a hyper-variable locus to monitor diversity within strains of the same pathotype. The *pspB* (PD1208) locus, encoding a serine protease, was selected and analyzed. It was observed that tandem repeat sequences in *pspB* locus were highly variable from strain to strain. The biological significance of this hyper-variation is unknown. We also evaluated a simple sample preparation method for template DNA. The pulverized freeze-dried tissue PCR (PFT-PCR) test was compared to the “gold standard” pathogen isolation method. Our results indicated that PFT-PCR had a high predictive value (90.8%) for true positive samples, but a low predictive value for true negative results (29.7%), indicating that a PFT-PCR result is best suited to confirm the presence of *Xf* in a sample.

INTRODUCTION

The complete sequencing of *Xylella fastidiosa* (*Xf*) genome and their availability allow easy access to every genomic locus of the pathogen. With the availability of many primer design softwares in the Internet, it becomes highly feasible for many plant pathology laboratories to design PCR primers and explore their applications. These form the foundation for developing a genome based detection system for *Xf* using an array of primers from different loci on the platform of polymerase chain reaction (PCR). PCR is theoretically a highly sensitive and versatile technique for pathogen detection. A PCR experiment can be illustrated in the following formula:

$$\text{PCR SUCCESS} = \text{PRIMER} * \text{SAMPLE PREPARATION} \quad (1)$$

Where a PCR success is the function of utilization of primers designed based on genomic information and sample preparation that provides DNA template. Either factor is equally critical in affecting PCR outcome.

The evolution and nucleotide variation rates of each gene or genomic locus vary. Therefore, primers designed from different genomic loci have different value in evaluating the bacterial population diversity and usage to define *Xf* strains. Several specific PCR primer sets are currently available for *Xf* detection including the most thoroughly tested RST31/33 primer set (Minsavage, et al., 1994), derived from the RNA polymerase genomic locus, and those derived from 16S rRNA gene (Chen et al., 2005), an important taxonomical character for the description of *Xf* (Wells et al., 1987). These primers target the conserved genes. Variations in these gene sequences are closely associated with pathogen pathotypes. For example, in San Joaquin Valley of California, the 16S rDNA G-genotype (G-genotype) strains cause both Pierce’s disease (PD) of grapevine and almond leaf scorch disease (ALSD). The 16S rDNA A-genotype (A-genotype) strains cause only ALS. Few studies have been performed on the use of less conserved or highly variable loci to study *Xf*. Information from the more variable loci could facilitate our understanding of the bacterial pathogenicity and environmental adaptations.

In contrary to PCR primers, sample preparation methods have been subjected to much less vigorous evaluation. The most common PCR detection procedures for *Xf* detection involve DNA extraction to generate template DNA. However, this severely reduces the high throughput capacity of a PCR procedure. Efforts were made to simplify or omit the DNA extraction procedure by using expressed plant sap as PCR DNA template. However, the results were inconsistent. To address this problem, we previously reported the development of a procedure using pulverized freeze-dried almond tissues for PCR detection of *Xf* (Chen and Civerolo, 2005).

In this report, we present the results of our recent analyses on using primers from a hyper-variable locus to evaluate the population diversity of *Xf* strains within the same pathotype/genotype. We also evaluated the procedure using pulverized freeze-dried almond tissues for PCR (PFT-PCR) detection of *Xf*. These are part of our effort in developing a comprehensive genome sequence-based detection system for *Xf* strains important to California.