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Mutations in Type I and Type IV Pilus Biosynthetic Genes Affect Twitching Motility Rates in *Xylella fastidiosa*▽†

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Xylella fastidiosa **possesses both type I and type IV pili at the same cell pole. By use of a microfluidic device, the speed of twitching movement by wild-type cells on a glass surface against the flow direction of media was measured as 0.86 (standard error [SE], 0.04) μm min^{-1}. A type I pilus mutant (*fimA*) moved six times faster (4.85 [SE, 0.27] μm min^{-1}) and a *pilY1* mutant moved three times slower (0.28 [SE, 0.03] μm min^{-1}) than wild-type cells. Type I pili slow the rate of movement, while the putative type IV pilus protein PilY1 is likely important for attachment to surfaces.**

Xylella fastidiosa is a gram-negative, nonflagellated, plant-pathogenic bacterium that causes economically important diseases of many crops, including Pierce's disease of grapevine (15). The bacterium is limited to colonizing xylem vessels, the water (sap) conduits of plants, where it develops biofilms that block the flow of sap, culminating in disease symptoms (8). Recently, it was demonstrated that the type IV pilus-mediated twitching motility of the bacterium occurred predominantly against the direction of nutrient liquid flow in microfluidic devices (12). Corresponding basipetal intraplant spread of *X. fastidiosa*, i.e., against the flow direction of xylem sap, was observed in planta (12). Particularly interesting is the fact that *X. fastidiosa* is the only bacterial species, to our knowledge, that possesses both types of pili at the same cell pole: type IV pili (1.0 to 5.8 μm in length) and type I pili (0.4 to 1.0 μm in length) (12). This dual pilus configuration may confer advantages related to cell motility and biofilm development within the confines of xylem elements.

Twitching motility via type IV pili has been observed in a number of gram-negative bacteria (11). Cell movement occurs as type IV pili extend from the bacterial cell, attach to the substratum, and are then retracted into the cell through a depolymerization process. The repeated extension-retraction process results in cell motility across surfaces at 2 to 20 (or more) μm min^{-1}, depending on the bacterial species, the substratum conditions, and whether the cells are solitary or in "rafts" (3, 17, 18, 19). A number of genes and gene products associated with type IV pilus-mediated twitching motility have been identified in various bacterial species, including *Pseudomonas aeruginosa*, *Neisseria gonorrhoeae*, and *Myxococcus xanthus* (1, 4, 11). Mutations in these genes, especially those involved in pilus assembly and retraction, often completely eliminate twitching motility (10); however, mutations in genes such as those coding for the pilus-associated protein PilY1, a pilus adhesion protein identified in *P. aeruginosa* (2), are likely to have less profound effects on cell motility.

As noted above, *X. fastidiosa* possesses an abundance of shorter type I pili that are products of the *fimA* gene (10, 12). The *fimA* mutant produced very weak biofilms compared to wild-type *X. fastidiosa* and mutants that lack type IV pili yet possess type I pili (10). Meng et al. (12) showed that basipetal translocation in planta by the *fimA* mutant of *X. fastidiosa* was somewhat greater than that by the wild-type parent isolate and far greater than that by *pilB* or *pilQ* mutants lacking type IV pili. This observation raised the question of the dual role of the two pilus types on the same cell and at the same pole: do type I pili govern cell motility?

Most studies to date concerning analysis of twitching motility as it relates to gene disruptions in the *pil* or *fim* genes have been conducted under static conditions, using either liquid media or agar-like surfaces, the exception being the work reported by Meng et al. (12). Functional analyses of the regulatory genes associated with type I and type IV pili under conditions of flowing medium not only provide an opportunity to test the limits of cell motility but also better represent environments which many bacterial species inhabit, such as xylem vessels of plants, industrial water systems, and mammalian internal structures (e.g., the urinary tract) as well as catheters and other medical devices. We report here our analysis of type IV pilus-mediated twitching motility in *X. fastidiosa* as it relates to the influence of the presence of type I pili (*fimA*) as well as to a specific set of type IV pilus-related genes, in particular, *pilY1* and *pilB*. We demonstrate that mutations in these genes profoundly alter the speed of twitching motility in *X. fastidiosa*, particularly under flow conditions in microfluidic devices.

Cell motility under different flow rates. The speed at which wild-type *X. fastidiosa* cells (ATCC 700964) (20) travel via type IV pilus-mediated twitching movement against variable flow rates was examined. To accomplish this, microfluidic devices were fabricated using a channeled polydimethylsiloxane (Sylgard 184; Dow Corning, Midland, MI) body sandwiched between a glass microscope slide and a cover glass similar to that previously described (6, 12). In brief, the polydimethylsiloxane body was molded from a micropatterned silicon wafer bearing two parallel 80-μm-wide, 3.7-cm-long, and 50-μm-high ridges, separated by a 50-μm-wide groove (Fig. 1). Small-bore Teflon

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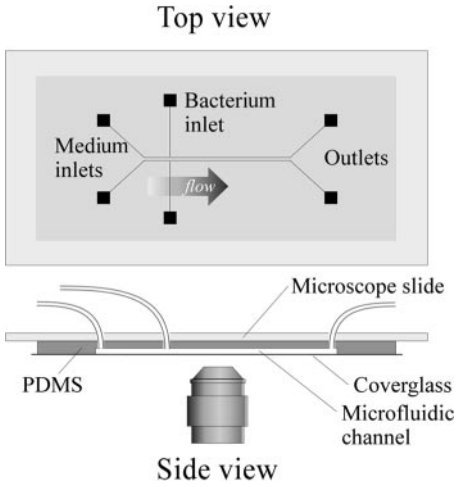

FIG. 1. Diagrammatic representation of the microfluidic device used to examine type IV pilus-mediated twitching motility in *X. fastidiosa* wild-type and mutant cells. PDMS, polydimethylsiloxane.

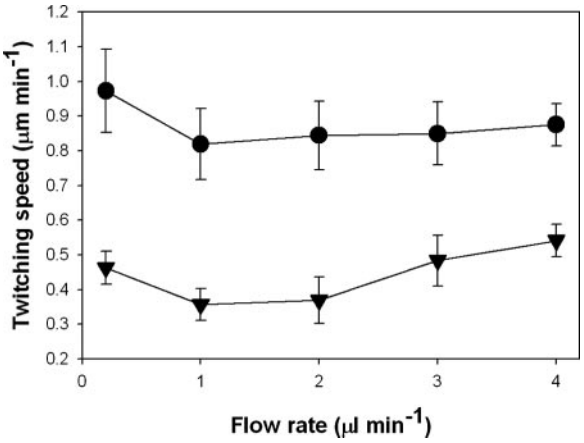

FIG. 2. Migratory speed of wild-type *X. fastidiosa* cells facilitated by type IV pilus-mediated twitching motility against various medium flow rates. The speed of movement was determined for two different scenarios: total distance traversed by cells in any direction (●) and distance traversed by cells in the direction of the *x* axis only (▼). Values represented in the graph correspond to means and SEs. Statistical analyses were performed with STATISTIX 8.0 software (Analytical Software, St. Paul, MN). The Kruskal-Wallis test was used whenever analysis-of-variance assumptions were violated. There were no differences among means at a *P* value of 0.05.

tubing (inside diameter, 0.022 in.; Weico, Edgewood, NY) was affixed to the inlets and outlets of the channels. Flow of PD2 nutrient medium (5) through the microfluidic channels was controlled with a syringe pump (Pico Plus; Harvard Apparatus, Holliston, MA) fitted with two 5-ml syringes, one for each channel. Once the cells attached to the glass surfaces of the microfluidic channels, medium flow was maintained at 0.2 μl min^{-1} for up to 10 h to ensure optimal conditions for cell-twitching motility, after which flow rates of 0.2 to 4 μl min^{-1} (equivalent to approximately 1×10^4 to 2×10^5 μm min^{-1}, as determined from visual measurements of 0.5-μm-diameter latex beads transported with the flow near the channel substratum) were maintained for at least 3 h. During these times, *X. fastidiosa* cell activities were observed microscopically and assessed using time lapse image recording every 30 s as previously reported (12). Thirty to 45 cells were tracked for each flow rate in 10 independent experiments ($n = 210$). To calculate the speed of twitching movement, the distance of displacement of cells was determined for (i) the length of the total path covered and (ii) the length along the *x* axis according to the starting and ending positions.

No significant differences ($P > 0.05$) according to the Kruskal-Wallis test were observed for the speed of movement of wild-type *X. fastidiosa* cells at different flow rates, for either the total path covered (0.86 [standard error {SE}, 0.04] μm min^{-1}) (all speed values are expressed as averages and SEs) or the displaced distance along the *x* axis (0.44 [SE, 0.02] μm min^{-1}) (Fig. 2). The insignificant differences in speed of movement by the bacteria for the range of flow rates used in this study (0.2 to 4 μl min^{-1}) reflect extremely small differences in the drag forces on the cells near the channel surface (6). These flow rates provided drag force large enough for the cells to align with the flow but small enough for the cells to remain attached. Thus, it was possible to combine all data from different flow rates. Subsequent calculations of twitching speeds of wild-type *X. fastidiosa* as well as the mutants (see below) were determined only for the length of the total path covered. Previous observations for rates of cell motility mediated by type IV pili in other bacterial species have been under static (no-flow) conditions (13, 17, 19). Speeds of twitching motility in *P. aeruginosa* have been reported as 19 (standard deviation, 12) μm min^{-1} (18) and 6 μm min^{-1} (17). Speeds of gliding motility of *M. xanthus* (associated with type IV pili) were reported to be 2 μm min^{-1} (3) and 6 μm min^{-1} (19). Neither *P. aeruginosa* nor *M. xanthus* have type I pili; thus, the speeds of these bacteria can be compared only to that of the *fimA* mutant of *X. fastidiosa*.

Twitching speed of pilus-defective mutants. *pil* and *fim* mutants of *X. fastidiosa* were studied under flow conditions to ascertain how pili influenced cell motility. Three mutants, a *fimA* (PD0062) mutant lacking short type I pili, a *pilB* (PD1927) mutant lacking longer type IV pili (12), and a *pilY1* (PD0023) mutant lacking the type IV pilus protein PilY1 (10) (previously notated as 6E11, 1A2, and TM14, respectively) were generated by random mutagenesis using an EZ::TN Tn*5* transposome system (7). For these mutants, flow conditions in microfluidic devices and analysis of cell movement were as described above for the wild-type isolate of *X. fastidiosa*. *fimA* and *pilY1* mutant cells were tracked at four and three different flow rates, respectively, in two independent experiments ($n =$ 40 and 35, respectively). *pilB* mutant cells lacking type IV pili were used as a control and were nonmotile (12). As with the wild-type *X. fastidiosa* cells, no significant differences ($P >$ 0.05) in twitching motility rates were observed for the *fimA* or *pilY1* mutants for the various flow rates; thus, data from the various experiments were combined for each mutant. The motility rate for the *fimA* mutant was 4.85 (SE, 0.27) μm min^{-1}, significantly faster than the 0.86 (SE 0.04) μm min^{-1} rate observed in wild-type cells, while the rate for the *pilY1* mutant was significantly lower, at 0.28 (SE, 0.03) μm min^{-1} (Fig. 3 and 4) (see the supplemental material and http://www.nysaes.cornell.edu/pp/faculty/hoch/movies/). Differences in twitching

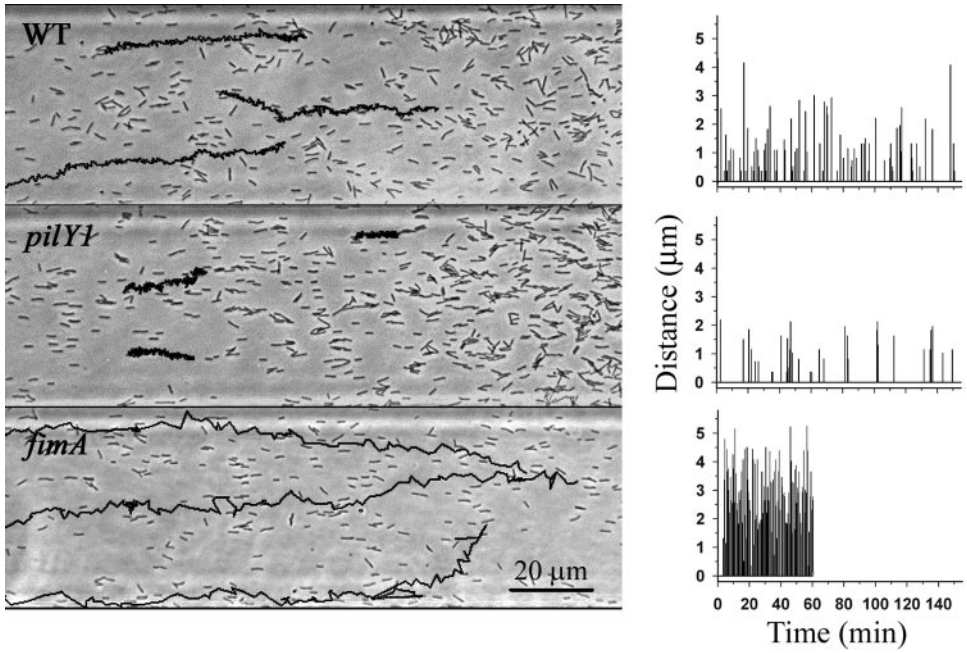

FIG. 3. Comparative paths of type IV pilus-mediated twitching movement of *X. fastidiosa* wild-type (WT) cells and *pilY1* and *fimA* mutants. Black traces correspond to migratory paths (right to left) against medium flow (left to right) for three representative cells of each cell type over the same period of time. Each line within the graphs (right) indicates the distance displaced for each 30-s interval by twitching movement for one representative cell of each cell type. Note that *fimA* mutants moved greater distances than the length of the field of view during the time interval shown. Total time, 150 min. Movies of this figure are available in the supplemental material and at http://www.nysaes.cornell.edu/pp/faculty/hoch/movies/.

movement are further exemplified in the graphs in Fig. 3, which relate displacement distances with time for individual cells. Wild-type cells moved more or less constantly during the time period, and *pilY1* mutant cells moved more sporadically, with frequent "stop" intervals (distance = 0 μm). *fimA* mutant cells moved greater distances constantly and after about 60 min moved out of the field of view.

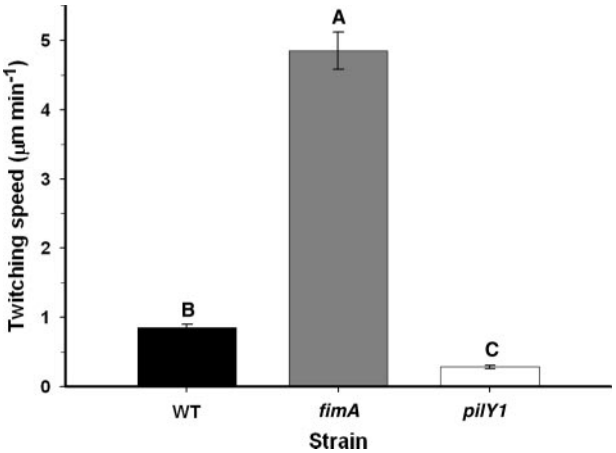

FIG. 4. Comparative migratory speeds of type IV pilus-mediated twitching movement of *X. fastidiosa* wild-type (WT) cells and *pilY1* and *fimA* mutants. Values shown are means and SEs from 2 (*pilY1* and *fimA* mutants) and 10 (WT) independent experiments. Analysis of variance of the log-transformed data showed significant differences among treatments ($P < 0.0001$). Letters indicate significant differences ($P = 0.05$) by Fisher's protected least-significant-difference test.

Cyro-scanning electron microscopy of frozen-hydrated wild-type *X. fastidiosa* cells confirmed the polar presence of both type I and type IV pili (Fig. 5a). Electron microscopy revealed that the *fimA* mutant bears only type IV pili (Fig. 5b) (12). The *pilY1* mutant has the complete complement of type I and type IV pili with lengths similar to those observed in wild-type bacteria (Fig. 5c) (10). Meng et al. (12) showed that the *fimA* mutant migrated greater distances against plant xylem transpiration-mediated sap flow than did wild-type cells, an observation now explained by the microfluidic device results obtained in this study for the *fimA* mutant and wild-type isolate. Our observations, along with the finding by De La Fuente et al. (6) that type I pili are extremely adherent to surfaces, indicate that their presence slows type IV pilus-mediated migration and probably plays a role in bacterial biofilm formation (10) in the xylem environment under sap flow. PilY1, a protein first described for *P. aeruginosa* with homology to PilC2 of *N. gonorrhoeae* (2), has been found in both cell membrane and fimbrial fractions. It has been hypothesized that the protein serves as an "adhesin" at the tip of the type IV pilus (9, 16) and as an assembly platform for type IV pili (2, 14). Indeed, the slower twitching motility of the *X. fastidiosa pilY1* mutant would be consistent with a role for a pilus adhesin protein. Although the relationship between the speed of twitching motility and plant disease expression by *X. fastidiosa* is yet to be fully demonstrated, preliminary observations indicate that plants inoculated with faster-migrating mutants (e.g., *fimA*) develop symptoms earlier, while plants inoculated with slower-migrating mutants (e.g., *pilY1*) are delayed in symptom expression.

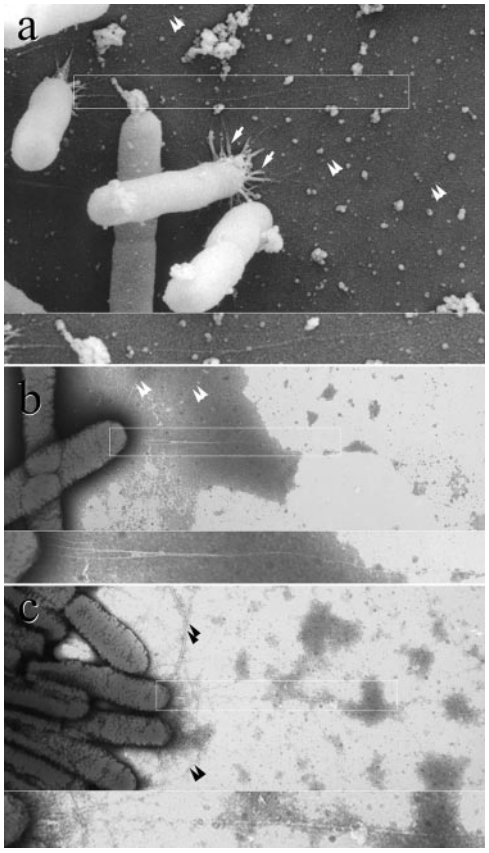

FIG. 5. Electron micrographs of *X. fastidiosa* cells. (a) Cyro-scanning electron microscopy micrograph of frozen-hydrated wild-type cells depicting an abundance of short type I pili at the cell pole in contact with the substratum and fewer long type IV pili. (b, c) Transmission electron microscopy micrographs of *fimA* (b) and *pilY1* (c) mutant cells negatively stained with phosphotungstic acid. Only type IV pili are present on the *fimA* (b) mutant cells, whereas both pilus types are present on the *pilY1* (c) mutant cells. Single arrows, type I pili; double arrowheads, type IV pili (which are also noted within the boxed area enlarged below each panel).

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REFERENCES

1. **Alm, R. A., and J. S. Mattick.** 1997. Genes involved in the biogenesis and function of type-4 fimbriae in *Pseudomonas aeruginosa*. Gene **192:**89–98.
2. **Alm, R. A., J. P. Hallinan, A. A. Watson, and J. S. Mattick.** 1996. Fimbrial biogenesis genes of *Pseudomonas aeruginosa*: *pilW* and *pilX* increase the similarity of type 4 fimbriae to the GSP protein-secretion systems and *pilY1* encodes a gonococcal PilC homologue. Mol. Microbiol. **22:**161–173.
3. **Blackhart, B. D., and D. R. Zusman.** 1985. "Frizzy" genes of *Myxococcus xanthus* are involved in control of frequency of reversal of gliding motility. Proc. Natl. Acad. Sci. USA **82:**8767–8770.
4. **Burrows, L. L.** 2005. Weapons of mass retraction. Mol. Microbiol. **57:**878–888.
5. **Davis, M. J., W. J. French, and N. W. Schaad.** 1981. Axenic culture of the bacteria associated with phony disease of peach and plum leaf scald. Curr. Microbiol. **6:**309–314.
6. **De La Fuente, L., E. Montanes, Y. Meng, Y. Li, T. J. Burr, H. C. Hoch, and M. Wu.** 2007. Assessing adhesion forces of type I and type IV pili of *Xylella fastidiosa* using a microfluidic flow chamber. Appl. Environ. Microbiol. **73:** 2690–2696.
7. **Guilhabert, M. R., L. M. Hoffman, D. A. Mills, and B. C. Kirkpatrick.** 2001. Transposon mutagenesis of *Xylella fastidiosa* by electroporation of Tn5 synaptic complexes. Mol. Plant-Microbe Interact. **14:**701–706.
8. **Hopkins, D. L.** 1989. Xylem-limited bacterial pathogen of plants. Annu. Rev. Phytopathol. **27:**271–290.
9. **Kirchner, M., and T. F. Meyer.** 2005. The PilC adhesin of the *Neisseria* type IV pilus—binding specificities and new insights into the nature of the host cell receptor. Mol. Microbiol. **56:**945–957.
10. **Li, Y., G. Hao, C. D. Galvani, Y. Meng, L. De La Fuente, H. C. Hoch, and T. J. Burr.** 2007. Type I and type IV pili of *Xylella fastidiosa* affect twitching motility, biofilm formation, and cell-cell aggregation. Microbiology **153:**719–726.
11. **Mattick, J. S.** 2002. Type IV pili and twitching motility. Annu. Rev. Microbiol. **56:**289–314.
12. **Meng, Y., Y. Li, C. D. Galvani, G. Hao, J. N. Turner, T. J. Burr, and H. C. Hoch.** 2005. Upstream migration of *Xylella fastidiosa* via pilus-driven twitching motility. J. Bacteriol. **187:**5560–5567.
13. **Merz, A. J., M. So, and M. P. Sheetz.** 2000. Pilus retraction powers bacterial twitching motility. Nature **407:**98–102.
14. **Nassif, X., J. L. Beretti, J. Lowy, P. Stenberg, P. O'Gaora, J. Pfeifer, S. Normark, and M. So.** 1994. Roles of pilin and PilC in adhesion of *Neisseria meningitidis* to human epithelial and endothelial cells. Proc. Natl. Acad. Sci. USA **91:**3769–3773.
15. **Purcell, A. H., and D. L. Hopkins.** 1996. Fastidious xylem-limited plant pathogens. Annu. Rev. Phytopathol. **34:**131–151.
16. **Rudel, T., I. Scheuerpflug, and T. F. Meyer.** 1995. Neisseria PilC protein identified as type-4 pilus tip-located adhesin. Nature **373:**357–359.
17. **Semmler, A. B., C. B. Whitchurch, and J. S. Mattick.** 1999. A re-examination of twitching motility in *Pseudomonas aeruginosa*. Microbiology **145:**2863–2873.
18. **Skerker, J. M., and H. C. Berg.** 2001. Direct observation of extension and retraction of type IV pili. Proc. Natl. Acad. Sci. USA **98:**6901–6904.
19. **Sun, H., D. R. Zusman, and W. Shi.** 2000. Type IV pilus of *Myxococcus xanthus* is a motility apparatus controlled by the *frz* chemosensory system. Curr. Biol. **10:**1143–1146.
20. **Van Sluys, M. A., M. C. de Oliveira, C. B. Monteiro-Vitorello, C. Y. Miyaki, L. R. Furlan, L. E. Camargo, A. C. da Silva, D. H. Moon, M. A. Takita, E. G. Lemos, M. A. Machado, M. I. Ferro, F. R. da Silva, M. H. Goldman, G. H. Goldman, M. V. Lemos, H. El-Dorry, S. M. Tsai, H. Carrer, D. M. Carraro, R. C. de Oliveira, L. R. Nunes, W. J. Siqueira, L. L. Coutinho, E. T. Kimura, E. S. Ferro, R. Harakava, E. E. Kuramae, C. L. Marino, E. Giglioti, I. L. Abreu, L. M. Alves, A. M. do Amaral, G. S. Baia, S. R. Blanco, M. S. Brito, F. S. Cannavan, A. V. Celestino, A. F. da Cunha, R. C. Fenille, J. A. Ferro, E. F. Formighieri, L. T. Kishi, S. G. Leoni, A. R. Oliveira, V. E. Rosa, Jr., F. T. Sassaki, J. A. Sena, A. A. de Souza, D. Truffi, F. Tsukumo, G. M. Yanai, L. G. Zaros, E. L. Civerolo, A. J. Simpson, N. F. Almeida, Jr., J. C. Setubal, and J. P. Kitajima.** 2003. Comparative analyses of the complete genome sequences of Pierce's disease and citrus variegated chlorosis strains of *Xylella fastidiosa*. J. Bacteriol. **185:**1018–1026.