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A Short Protocol for Gene Knockout and Complementation in *Xylella fastidiosa* Shows that One of the Type IV Pilin Paralogs (PD1926) Is Needed for Twitching while Another (PD1924) Affects Pilus Number and Location

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ABSTRACT Twitching motility is one of the major virulence factors of the plant-pathogenic bacterium *Xylella fastidiosa*, and it is mediated by type IV pili (TFP) that are present at one of the cell poles. Genome analysis of *X. fastidiosa* showed the presence of at least four paralogs of the gene *pilA*, which encodes the TFP major pilin subunit. However, whether all of these paralogs have a functional role in TFP structure and function is unknown. Here, using a short and reliable protocol based on overlap extension PCR and natural transformation, deletion mutants of two *pilA* paralogs (*pilA1* PD1924 and *pilA2* PD1926) were generated in two *X. fastidiosa* subsp. *fastidiosa* strains, WM1-1 and TemeculaL, followed by assessment of twitching motility and biofilm formation. Deletion of *pilA2* caused loss of twitching motility, whereas deletion of *pilA1* did not influence twitching motility but caused hyperpiliation and extended distribution of TFP along the sides of the cell. Loss of twitching motility due to *pilA2* deletion was restored when a wild-type copy of the *pilA2* gene was added at a neutral site in the genome of mutants in both wild-type backgrounds. This study demonstrates that PCR templates generated by overlap extension PCR can be successfully used to rapidly generate gene knockouts and perform genetic complementation in *X. fastidiosa*, and that twitching motility in *X. fastidiosa* is controlled by regulating the transcription of the major pilin subunit, *pilA2*.

IMPORTANCE The bacterial plant pathogen *Xylella fastidiosa* causes incurable diseases in multiple hosts, including grape, citrus, and blueberry. Historically restricted to the Americas, it was recently found to cause epidemics in olives in Italy and to infect other hosts in Europe and Asia. In this study, we report a short protocol to create deletion and complemented mutants using fusion PCR and natural transformation. We also determined the distinct function of two pilin paralogs, the main structural component of TFP involved in twitching motility, which allows this bacterium to move inside the xylem vessels against the flow. One of the paralogs is needed for twitching movement, whereas the other does not have an effect on motility but influences the number and position of TFP. Since twitching motility is fundamental for the virulence of this xylem-limited bacterium, this study contributes to the understanding of the regulation of virulence by this pathogen.

KEYWORDS *Xylella fastidiosa*, fastidious prokaryote, hyperpiliation, mutagenesis, natural transformation systems, paralog, *pilA*, pilin, twitching

Xylella fastidiosa is a Gram-negative, xylem-limited, insect-vectored, plant-pathogenic bacterium causing incurable diseases and substantial economic losses to the production of grapevines, citrus, coffee, plum, and almond (1, 2). Pierce's disease (PD) of grapevines in the United States and citrus variegated chlorosis (CVC) in Brazil, two of the most impactful diseases caused by *X. fastidiosa*, are major limiting factors in the production of

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these crops in those countries (2). Xylem sap-feeding insect vectors, including sharpshooters and spittlebugs, transmit and inoculate the bacterial cells into xylem vessels, where the cells attach to the surface, form biofilms, and move along and across the xylem vessels (3–5). The severity of disease symptoms depends on the extent of xylem vessel occlusions produced by bacterial aggregates (3). Therefore, cell aggregation and systemic colonization of the xylem vessels are considered the major determinants of pathogenicity in *X. fastidiosa*.

X. fastidiosa cells are nonflagellated but possess two types of pili at one of their cell poles. Short type I pili (0.4 to 1.0 μm) are involved in cell attachment and biofilm formation, and long type IV pili (TFP) (1.0 to 5.8 μm) are involved in twitching motility (6–8). Twitching motility involves flagellum-independent translocation of cells on a moist surface, facilitated by extension, tethering, and retraction of TFP (9). It was demonstrated in *X. fastidiosa* that a mutant lacking type I pili (*fimA* mutant) was biofilm deficient but twitching enhanced, whereas the opposite phenotype was true for mutants lacking TFP (6–8). Moreover, mutants that were twitching enhanced traveled further upstream from the point of inoculation in grapevines (8). In addition, *X. fastidiosa* wild-type (WT) strains that lacked or exhibited reduced twitching motility (10) had less severe disease symptoms in the model plant tobacco and in the natural host blueberry (11, 12). A polygalacturonase gene mutant that is compromised in lateral movement due to its inability to degrade xylem pit membranes was avirulent in grapevines (4) and was found to be twitching deficient in another study (13). Furthermore, seven transposon mutants, including a mutant of hemagglutinin that mediates cell-cell aggregation, showed hypervirulent phenotypes and faster movement than that of wild-type strains in grapevines (14). Previous studies demonstrated the role of cell-cell communication system involving a diffusible signaling factor (DSF) in regulating host colonization, movement, and biofilm formation in *X. fastidiosa* (15). Interestingly, it was shown that a mutant that was unable to produce DSF was hypervirulent to grapevines (15) and had increased expression of several TFP genes compared to that of the wild-type strain (16). Together these observations suggest that motility of cells is one of the important virulence factors of *X. fastidiosa*, although this has not been directly demonstrated for this bacterium. Nevertheless, involvement of TFP and twitching motility in host colonization and virulence was demonstrated for other Gram-negative bacteria, including human pathogens, such as *Pseudomonas aeruginosa* (17), *Neisseria meningitidis* (18), *Neisseria gonorrhoeae* (19), and plant pathogens such as *Ralstonia solanacearum* (20), *Acidovorax citrulli* (21), *Xanthomonas citri* (22), and *Pseudomonas syringae* (23, 24). TFP were also shown to contribute to other bacterial functions like biofilm formation, electron transfer (9, 25), and natural DNA transformation (26), in addition to twitching motility.

TFP are flexible, filamentous structures mainly composed of polymers of a single pilin protein subunit that is encoded by *pilA* gene(s); however, multiple protein components are involved during TFP biogenesis and function. For example, in *P. aeruginosa*, where twitching motility and TFP have been characterized in detail, approximately 40 genes are required for the function of TFP, including genes for the pilin subunit, pilin assembly and retraction, and regulatory genes (9). Genomes of *X. fastidiosa* contain numerous genes (27, 28) that share signature motifs and properties of well-described pilus genes. The role in *X. fastidiosa* of a few of these genes in TFP production and twitching motility was demonstrated in previous studies (6–8, 29, 30). At least six open reading frames (ORFs) that encode characteristic pilin subunits (*pilA* homologs) were predicted in the genome of CVC strain 9a5c and one of the ORFs was found to be regulated by RpoN, the alternative sigma 54 (σ^{54}) factor (31). At least four homologs of the *pilA* ORFs are also present in the grapevine strain Temecula1 (28). Protein products of two of these homologs, designated PilA1 (PD1924) and PilA2 (PD 1926) in previous studies (16, 32), share functional features of the TFP pilin subunit (9). Whole-transcriptome analysis by transcriptome sequencing (RNA-Seq) showed that both of these *pilA* paralogs are expressed in Temecula1, although the expression of *pilA2* was greater than that of *pilA1* (32). However, the exact role of these *pilA* paralogs in *X. fastidiosa* remains poorly understood, and whether both or only one of them has

a major functional role in TFP formation and twitching motility is unknown. A clear understanding of this issue could help researchers in recognizing the regulation of TFP formation and twitching motility in *X. fastidiosa*.

The objective of this study was to characterize the function of the two pilin paralogs, *pilA1* (PD1924) and *pilA2* (PD1926), in two *Xylella fastidiosa* subsp. *fastidiosa* strains, WM1-1 and TemeculaL. We found that while *pilA1* was not necessary for twitching motility (unlike *pilA2*), mutation on this gene generated hyperpiliated cells. For this study, we designed a new protocol for mutagenesis that simplifies and expedites the protocols used before, which relied on either electroporation (30, 33) or natural competence (34, 35) to introduce plasmids for homologous recombination. The new protocol for gene deletion and complementation used in this study utilizes overlap extension PCR and natural genetic transformation of *X. fastidiosa*. Results show that this method is reliable and can be used to rapidly generate mutants in *X. fastidiosa*.

RESULTS

Sequence analysis of *pilA* paralogs and their promoter. Based on gene expression analysis, a pilin paralog (XF2542) was predicted to be the major pilin of *Xylella fastidiosa* subsp. *pauca* strain 9a5c (31). A BLAST search using the 9a5c sequence of this paralog as query against the Temecula1 genome showed PD1926 (*pilA2*) as the closest homologue, with 98% coverage and 87% amino acid sequence identity, while a second paralog PD1924 (*pilA1*) had 97% coverage and 65% identity. On pairwise alignment, the two paralogs of Temecula1 (PD1924 and PD1926) were 68% identical by nucleotide sequence and 62% identical by amino acid sequence. They are not part of any operons (32), and they are separated by a hypothetical protein (PD1925) that is transcribed in a different strand. However, both contain all the characteristic features of a pilin subunit, including a leader peptide, cleavage site, N-terminal hydrophobic region, and pilin-cytoplasmic, transmembrane, and extracellular domains (data not shown). On comparing regulatory regions in the promoter based on the regions predicted by the previous study on *X. fastidiosa* subsp. *pauca* 9a5c (31), putative binding sites of the alternative sigma factor σ^{54} were identified in the promoter of PD1926 in both Temecula1 and WM1-1 (see Fig. S1 in the supplemental material). Furthermore, sequences of the coding regions and promoters of these pilin genes (*pilA1* and *pilA2*) were identical in WM1-1, TemeculaL, and the reference strain Temecula1 (data not shown). Therefore, DNA sequence analyses suggest that the *pilA* paralog encoded by PD1926 (*pilA2*) is the functional pilin in *X. fastidiosa* subsp. *fastidiosa* strains WM1-1 and TemeculaL, and its expression is predicted to be under σ^{54} regulation.

Generation of mutants by overlap PCR and natural transformation. Transformants were successfully generated from all overlap PCR-generated homologous templates that targeted two pilin genes, as well as two additional *X. fastidiosa* genomic regions, in the strains WM1-1 and TemeculaL (Table 1). The number of transformants ranged from 6.2 $\pm$ 3.4 to 103.6 $\pm$ 9.5 per 100 μl of recipient cell culture, depending on the construct and the strain used (Table 1). The estimated transformation frequency (ratio of transformant CFU to total viable CFU) with the protocol used here is $\sim 10^{-4}$ to 10^{-6} per recipient cell, based on our previous characterization (10, 13) of the same strains using plasmid DNA and heat-killed donor cells. The mutagenesis protocol was not optimized, since it worked satisfactorily (i.e., at least a few recombinant colonies were recovered on every experiment), but we observed that some important steps to consider in the success of this method for *X. fastidiosa* are as follows: (i) overlapping regions must be at least 21 bp between the two fragments, as regions shorter than this did not result in successful fusion of the PCR products (data not shown), and (ii) the growth stage of the cells, as well as the growth medium, influences the rate of natural transformation, as demonstrated in our previous studies (10, 13). We therefore suggest performance of natural transformation at the logarithmic growth phase and use of Pierce's disease 3 (PD3) (36) or PD2 medium in solid agar condition (36). Periwinkle wilt (PW) medium (37) and *X. fastidiosa* medium (XFM) (38) could be used without bovine

TABLE 1 PCR transformants and twitching phenotypes of mutants generated with various PCR templates

Overlap PCR region (construct)[a]	Recipient strain/mutant	Mutation designation	No. of transformants (CFU ± SE)[b]	Mutant phenotype[c]
pilA1 (1924)	WM1-1	*pilA1*W	53.4 ± 15.9	Twitching ~ to WT
	TemeculaL	*pilA1*T	35.6 ± 20.7	Twitching ~ to WT
pilA2 (1926)	WM1-1	*pilA2*W	27.6 ± 4.5	Twitching deficient
	TemeculaL	*pilA2*T	6.2 ± 3.4	Twitching deficient
	pilA1 WM1-1	doubleW	19 ± 5	Twitching deficient
	pilA1 TemeculaL	doubleT	103.6 ± 9.5	Twitching deficient
pilA2 complementation	*pilA2* WM1-1	*pilA2*CW	20 ± 3.7	Twitching ~ to WT
	pilA2 TemeculaL	*pilA2*CT	8.5 ± 3.5	Twitching ~ to WT
Upstream *pilQ* (1691)	WM1-1	*UpilQ*W	51 ± 24.8	Twitching ~ to WT
mrcA (1695)	WM1-1	*mrcA*W	57 ± 29.2	Twitching deficient

[a]Name indicates target gene used to design homologous regions. All constructs include an antibiotic resistance cassette used for selection (see Materials and Methods).
[b]Values indicate the mean number of transformant cells ± standard error (SE), obtained from a 0.1-ml cell suspension from three replications.
[c]Twitching ~ to WT, twitching similar to the wild-type value.

serum albumin (BSA) supplementation. The basic steps of the protocol are summarized in Fig. S2 in the supplemental material.

Growth curve and biofilm formation of *pilA* mutants. Growth curves (Fig. 1) show that growth of strains was similar for the mutants and wild-type strains. Slight growth increases were observed in the stationary phase for the *pilA2*W mutant (Fig. 1A) and *pilA1*T mutant (Fig. 1B). But these differences are probably due to differences in surface attachment of the mutant cells to the microtiter plate. Biofilm formation increased in both *pilA* mutants compared to that of the wild-type strains (Fig. 2). Similar tendencies were observed in biofilm formation with both TemeculaL and WM1-1 backgrounds, where the increase was significant ($P = 0.0004$ [*pilA1*W], $P < 0.001$ [*pilA*2W, *pilA1*T, and *pilA2*T]) (Fig. 2). Both double mutants had significantly ($P < 0.0001$) higher biofilm formation than that of wild-type strains, and higher than that of the single *pilA* mutants (Fig. 2). Complementation of *pilA2* (*pilA2*C) in TemeculaL reduced biofilm formation to the level of the wild type ($P > 0.05$) (Fig. 2).

Twitching motility and pilus formation by wild type and mutants. Formation of colony fringe was observed to assess twitching motility, as shown in Fig. 3. Both

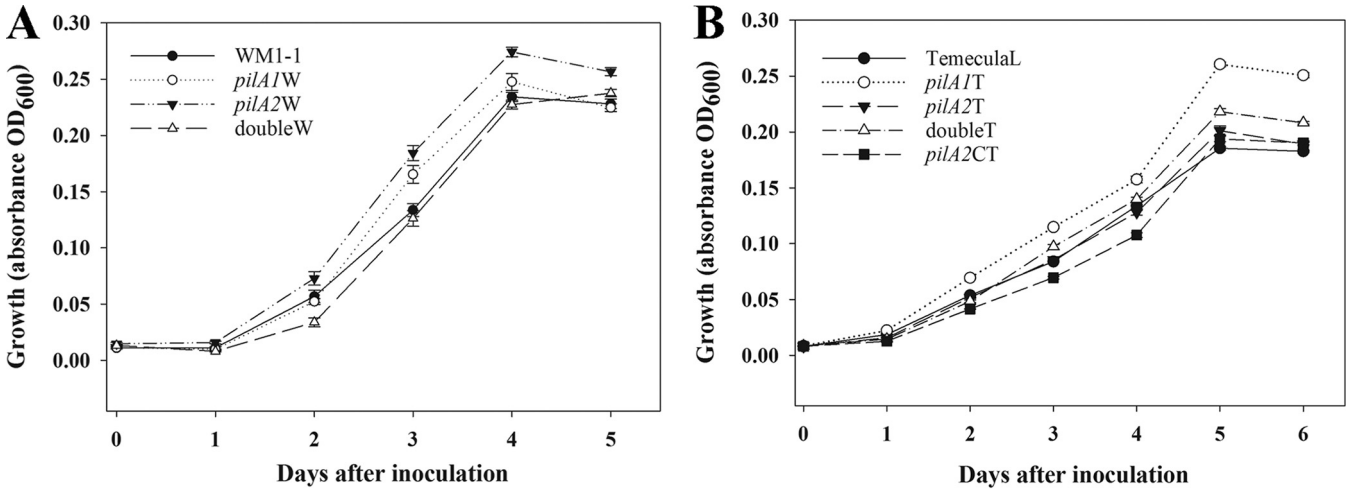

FIG 1 Growth curves of *Xylella fastidiosa* strains. Growth curves of wild-type *X. fastidiosa* WM 1-1 (A) and TemeculaL (B) strains with their corresponding mutants in *pilA1* and *pilA2*, double mutants, and complemented *pilA2* were assessed by measuring optical density at 600 nm (OD_{600}) every day for 5 to 6 days. Experiments were repeated independently three times. At least 6 wells per plate (and 1 to 3 plates per experiment) were used for each strain. *pilA2*CW was not used in these experiments.

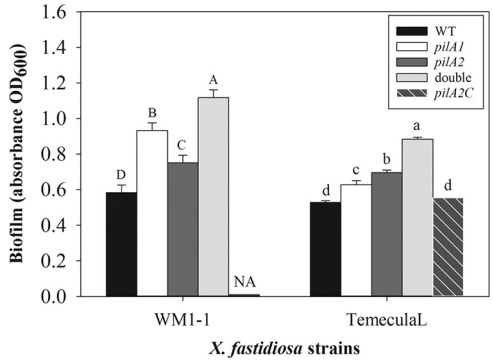

FIG 2 Biofilm formation of *Xylella fastidiosa* wild-type strains and mutants, as measured by crystal violet staining. Staining of biofilm was performed at the end of the growth curve experiment presented in Fig. 2. Experiments were repeated independently three times. At least 6 wells per plate (and 1 to 3 plates per experiment) were used for each strain. Data were analyzed in SAS 9.3 with PROC GLIMMIX. Error bars indicate standard error of the mean. Different uppercase (WM1-1 and its mutants) or lowercase (TemeculaL and its mutants) letters within each group indicate significant differences at 5% significance level. NA, nonavailable data.

wild-type and *pilA1* mutants of WM1-1 and TemeculaL displayed twitching motility, whereas the *pilA2* mutants were twitching deficient for both strains. As expected, double mutants of both wild-type strains were also twitching deficient (Fig. 3). Similar patterns of twitching were observed in media supplemented with 50% xylem sap (vol/vol) from an *X. fastidiosa*-susceptible grapevine variety, Chardonnay (data not shown).

To evaluate the effect of these mutations on pilus formation, wild-type and mutant cells were observed using transmission electron microscopy (TEM), as shown in Fig. 4. Pili were observed on one of the polar ends of wild-type *X. fastidiosa* cells. Compared with those of wild-type strains, pili of *pilA1* mutants were more abundant and not limited to the cell pole, as they were also found on the sides of the cells (Fig. 4). However, unlike in wild-type and *pilA1* mutant strains, long pili were not observed on *pilA2* mutants (Fig. 4). Average cell lengths of the wild type (WT) and mutants were compared, and no significant difference was observed (data not shown).

Complementation of *pilA2* restored twitching motility. *pilA2* mutants that were deficient in twitching motility were complemented using the wild-type copy of the *pilA2* ORF and its native promoter and terminator at the neutral site 1 (NS1) of *X. fastidiosa* TemeculaL and WM1-1 genomes, using the method shown in Fig. S2B. Both TemeculaL and WM1-1 mutants could be successfully complemented with the overlap fusion PCR product of the upstream-*pilA2*-downstream region PCR template. Several transformants (Table 1) were generated, and five were restreaked onto new PW plates supplemented with both antibiotics (kanamycin and chloramphenicol). Two of these colonies for each wild-type background were confirmed with PCR and sequencing for the insertion of the *pilA2* fragment in the neutral site. Twitching motility of five

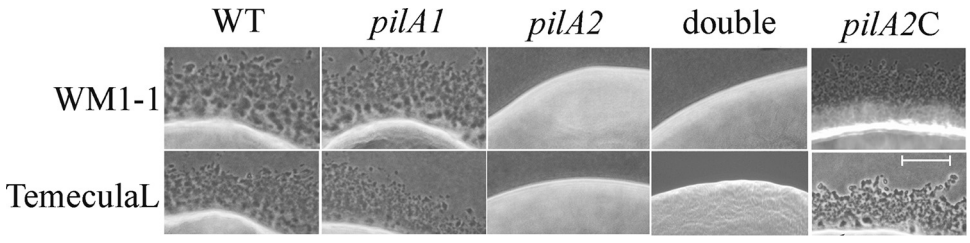

FIG 3 Twitching motility of wild-type, *pilA1* and *pilA2* mutants, and complemented *pilA2* mutant colonies. Twitching motility was observed for at least 10 independent transformants, with similar results obtained in all cases. For selected transformants for each background, observations were made at different time points. Bar (lower right panel), 100 μm.

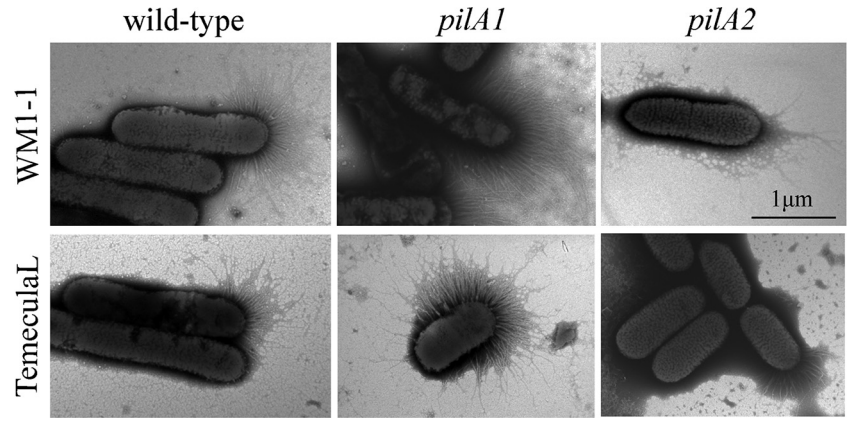

FIG 4 Transmission electron microscopy of pilus formation by *X. fastidiosa* wild-type and mutant strains. Wild-type and mutant cells were negatively stained by phosphotungstic acid. Images were captured at 10,000× magnification. Bar, 1 μm. Note the hyperpiliation and TFP distribution in both *pilA1* mutants.

transformant colonies selected for restreaking showed that complementation restored the twitching motility lost by *pilA2* deletion in both wild-type backgrounds (Fig. 3).

Expression of *pilA2* was higher than that of *pilA1*, and supplementation of BSA in PD3 reduced *pilA2* expression. Absence, and therefore lack of expression of the respective mutated gene (*pilA1* or *pilA2*), was confirmed for mutants in both wild-type backgrounds, as shown in Fig. S3 in the supplemental material, with *pilA*-specific primers. Expression of the two *pilA* paralogs in TemeculaL was compared among three different media using reverse transcription-quantitative PCR (qRT-PCR), and results are shown in Fig. 5A. Expression of *pilA1* was not changed by growth in PD3 supplemented with BSA (PD3+BSA) or grapevine variety Chardonnay sap (Sap). However, expression of *pilA2* was significantly reduced ($P = 0.006$) by addition of BSA to PD3. Expression of *pilA2* was significantly higher than that of *pilA1* in all media tested (Fig. 5B), suggesting that cells synthesize much more PilA2 protein than PilA1, which again supports *pilA2* being the major pilin subunit. Expression of *pilA2* was quantified in *pilA1* mutants of both WM1-1 and TemeculaL backgrounds (Fig. 5C), and in both cases an overexpression of *pilA2* was observed compared to expression in the respective wild-type strains, possibly explaining the greater number of TFP in *pilA1* mutants.

DISCUSSION

X. fastidiosa possesses TFP (6, 8), which mediates twitching motility, at one of the cell poles. Involvement of twitching motility in *X. fastidiosa* virulence was suggested by

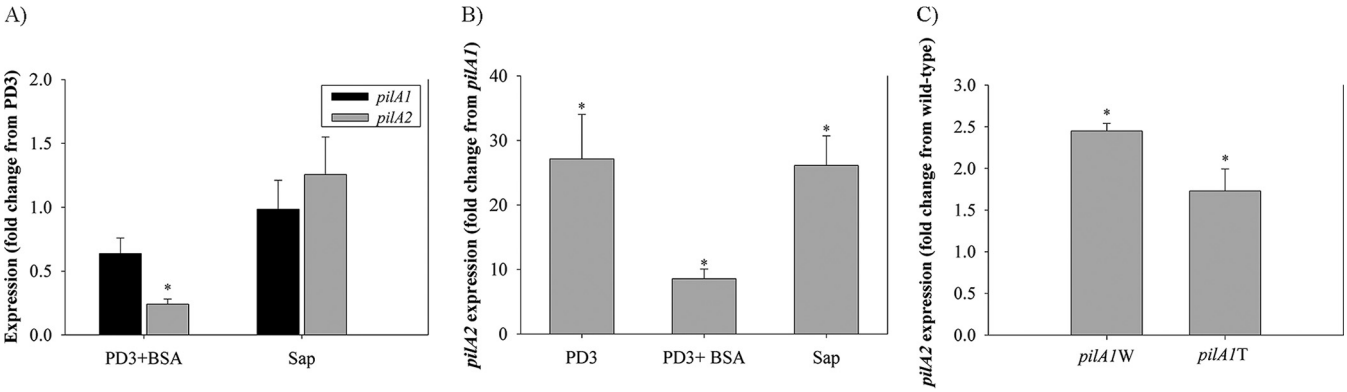

FIG 5 Expression analysis of the two *pilA* paralogs of TemeculaL and WM1-1 strains. For panels A and B, only TemeculaL and its mutants were used. (A) Results are expressed as fold change of *pilA* gene expression in cells cultured in PD3 medium supplemented with 3% BSA (PD3+BSA) and 100% grapevine variety Chardonnay sap (Sap) relative to expression in PD3. (B) Fold change in expression of *pilA2* relative to that of *pilA1* for the same cultures under three different media. (C) Expression of *pilA2* in *pilA1* mutants in both wild-type backgrounds (WM1-1 and TemeculaL) in PD3 media. Gene expressions was normalized by using *nuoA* as the endogenous control. An asterisk indicates significant difference in fold change, as compared by one-sample *t* test (fold change $\neq 1$; $P < 0.05$). Error bars indicate standard errors of mean in fold change from three independent replicates of one experiment.

indirect evidence, since mutants lacking TFP (due to mutations in *pilB* and *pilQ* genes) were deficient in twitching motility and had reduced colonization of grapevines (remaining close to the inoculation point) compared to that of the wild type (8). But since disease symptoms were not assessed in that study, a conclusion on the direct effect of twitching motility on virulence was not provided. The role in *X. fastidiosa* of some TFP-related genes, such as *pilB*, *pilO*, *pilQ*, *pilR*, and *pilY1*, in TFP biogenesis and twitching motility was demonstrated in previous studies (6–8, 29, 30). Moreover, environmental signals, such as calcium (30), pectin (39), and chitin (40), were shown to transcriptionally regulate twitching motility genes. Other studies demonstrated the involvement of chemotaxis-related genes (Pil-Chp) in regulating twitching motility and expression of TFP genes (29, 41). But there have been no studies in *X. fastidiosa* (besides the mentioned study in *X. fastidiosa* subsp. *pauca*; see reference 31) on the role of the major pilin(s) of TFP, a fundamental protein for this appendage.

In the present study, the role in twitching motility of two paralogs of type IV pilin (PD1924 and PD1926) was assessed. Despite *X. fastidiosa* genomes possessing several genes encoding pilin subunits (31) and at least two of these paralogs possessing similar structural features in the genomes of strains used in this study, the finding that only one of the *pilA* paralog (PilA2, PD1926) is essential for twitching motility was surprising. Of all the mutants generated, *pilA2* and *pilA1pilA2* double mutants lost twitching motility. Motility was restored in *pilA2* mutants when a copy of *pilA2* gene was reinserted at a different location in the genome. Interestingly, the *pilA1* mutants showed similar twitching motility to that of the wild types, although biofilm formation was increased in these mutants, suggesting a different role of PilA1. By electron microscopy, we were able to detect the formation of profuse TFP, which were distributed on the sides of the *pilA1* mutant cells, as opposed to the polar location in wild-type cells. Moreover, expression analysis indicated that higher production of *pilA2* is detected in *pilA1* mutants, which may explain the hyperpiliation observed in the *pilA1* mutants and the consequent increase in biofilm formation, since TFP is known to be involved in this process (24). The molecular basis for TFP regulation of localization and the number of appendages formed has not been completely elucidated. In the case of other appendages, such as flagella, proteins FlhF and FlhG in various bacteria are known to be involved in assembly and regulation of number and localization of these structures (42). In *Caulobacter crescentus*, polar localization of pili was shown to involve a two-component signal transduction histidine-kinase, PleC (43) and PodJ (44); but mutations in those proteins render pilus-less cells, a different phenotype than the one observed here. In *P. aeruginosa*, the membrane-associated polar organelle coordinator (Poc) complex regulates localization of flagellum and TFP (45), and mutations in their components led to random localization of these structures and lack of twitching motility. These phenotypes are different from the ones observed here, namely hyperpiliation and extended localization without losing the ability of twitching motility. The putative role of *pilA1* in regulating TFP localization needs to be further investigated.

Previous studies with *X. fastidiosa* (30, 46–48) have considered *pilA1* paralog in their expression analysis to determine the influence of various environmental parameters on twitching motility, but our results suggest that *pilA2* may be a better target to assess twitching in this bacterium. Expression analysis by RNA-Seq in our previous study (32) and by qRT-PCR in this current study showed that *pilA2* is expressed at a significantly higher level than that of *pilA1*. Moreover, the closest homologue of *pilA2* was demonstrated to be regulated differently by a σ^{54} factor in a *X. fastidiosa* subsp. *pauca* strain (31). Regulatory elements of σ^{54} in the *pilA2* promoter region of *X. fastidiosa* subsp. *fastidiosa* strains WM1-1 and TemeculaL used in this study were found by sequence comparison with the promoter of the *X. fastidiosa* subsp. *pauca* strain. Previous studies in *P. aeruginosa* have demonstrated that mutants defective in σ^{54} protein production were nonmotile and unable to produce TFP (49) and flagella (50). Furthermore, expression of type IV pilin was demonstrated to be regulated by σ^{54} and a PilR/PilS two-component regulatory system in a variety of other bacterial species (51–54). In *X. fastidiosa*, a mutant of the *pilR* gene, the response regulator of the PilR/PilS system, was

deficient in twitching (7). Therefore, the findings from previous studies that one of the *pilA* paralogs is differentially expressed in a RpoN mutant (31) and that the mutant of predicted σ^{54} activator is deficient in twitching (7), and the results of the present study that the pilin paralog suggested to be under σ^{54} regulation is twitching deficient, suggest that type IV pilin production in different *X. fastidiosa* strains may be regulated by σ^{54}, but this needs to be further studied.

In addition to its involvement in the regulation of genes involved in motility, the primary role of σ^{54} in various bacteria was described to be in nitrogen assimilation under nitrogen-limited conditions (55–57). Interestingly, in previous studies (13, 58), supplementation of a nitrogen-rich compound, BSA, in the culture medium of *X. fastidiosa* significantly reduced twitching motility, and in the present study, BSA was shown to decrease the expression of the major pilin *pilA2*. Although it is not known if *X. fastidiosa* cells use BSA as a nitrogen source, we speculate that it could act as an environmental signal that regulates the activation of the σ^{54} factor, which then activates genes related to nitrogen assimilation and motility.

Although *X. fastidiosa* was the first plant-associated bacterium to have its genome sequenced (27), there is still limited understanding regarding its virulence factors. This is because, similarly to other bacteria, almost 40% of the proteins encoded by the genome do not have a known function assigned and are annotated as hypothetical proteins (28), and, moreover, *X. fastidiosa* has been a difficult pathogen for genetic manipulation studies (59, 60). Although transposon mutagenesis (4, 8) and homologous recombination protocols (15, 30, 33, 61) have been used, these protocols are time-consuming and inconsistent in efficiency. This is further complicated by the fastidious and slow-growing nature of the bacterium (62). In this study, we developed a rapid and simplified protocol of genetic manipulation using overlap extension PCR and natural transformation, since *X. fastidiosa* was shown to be capable of natural competence (10, 63). Using this method with careful planning, gene knockouts can be generated within a month from the start of the bacterial culture at −80°C, and this method does not require any plasmid vectors or steps such as restriction digestion, ligation, *Escherichia coli* transformation, and plasmid preparation. Only a few PCR steps are needed to generate an allelic exchange template that recombines with the recipient bacterium, inserting a marker in exchange for the target gene. Moreover, the same process was successfully used to generate complemented mutants of the knocked-out genes. We believe that this simple, rapid, and reliable technique will augment genetic and functional studies aimed at understanding the virulence mechanisms of *X. fastidiosa*. This method was used here to tag five genomic locations with a marker gene with successful transformation. Transformation of *X. fastidiosa* strains with linear DNA has been reported in a previous study (63), and overlap extension PCR fragments were introduced in other bacteria via electroporation (64, 65) and natural transformation (66). The protocol for *X. fastidiosa* presented here is a simplified and faster approach compared to the one mentioned above (66), and it should be useful for research in this fastidious prokaryote.

In our previous studies, we showed that twitching-deficient strains or mutants lost the ability of natural transformation (10, 13). In previous research by our group and others (10, 35), *X. fastidiosa* twitching-deficient mutants of *pilO*, *pilQ*, and *pilM* genes were not transformable, whereas another twitching-deficient mutant in *pilB* was transformable. In this study, however, we could naturally transform both twitching-deficient *pilA2* mutants. Therefore, we speculate that although higher twitching motility increases the rate of natural transformation (10), a partial, rather than the complete, set of TFP structures may be sufficient for natural transformation of *X. fastidiosa*.

MATERIALS AND METHODS

Bacterial strains, media, and culture conditions. *X. fastidiosa* subsp. *fastidiosa* strains WM1-1 (67) and TemeculaL (a variant of Temecula1) (28), isolated from infected vineyards in Georgia and California, respectively, were used in this study. Strains were cultured in PW (37) agar plates, modified by omitting phenol red and using 1.8 g · liter^{-1} of bovine serum albumin (BSA; Gibco Life Sciences Technology), for 1 week at 28°C from −80°C glycerol stock, restreaked onto new PW plates, and cultured for another week

before use. PD3 medium (36) was used for culturing and suspending cells in liquid. Whenever needed, antibiotics kanamycin (Km) and chloramphenicol (Cm) were used at concentrations of 30 and 10 μg · ml^{-1}, respectively.

DNA extraction and knockout template construction. For DNA extraction, cells cultured on PW agar plates were suspended in 400 μl of sterile Milli-Q water and saved at −20°C until use. DNA was extracted using Quick-DNA fungal/bacterial miniprep kit (Zymo Research) using the manufacturers' protocol. Bead beating for DNA extraction was performed for 5 min using a Mini-BeadBeater-96 cell disruptor (BioSpec Products).

Knockout templates targeting four genomic regions, according to the Temecula1 reference sequence, were constructed. The four regions were the *pilA1* coding region (PD1924), the *pilA2* coding region (PD1926), the upstream noncoding region of *pilQ* (PD1691), and the downstream region of *pilM* (PD1695) that disrupted the *mrcA* gene encoding penicillin-binding protein 1A. These templates were named construct 1924, construct 1926, construct 1691, and construct 1695, respectively. Constructs 1691 and 1924 contained a Km-resistant cassette, while the other two constructs, 1695 and 1926, contained Cm-resistant cassettes. For creating each knockout construct, approximately 0.7- to 1.2-kb upstream and downstream flanking regions of the targeted genomic region were PCR amplified using the corresponding UP_F/UP_R and Dn_F/Dn_R primer pairs, respectively (Table 2). For example, 1924_up_F and 1924_up_R were used to amplify the region upstream from PD1924 (*pilA1*). Cassettes encoding Km or Cm resistance were amplified using primer pairs Kan_F and Kan_R or Cm_F and Cm_R, respectively (Table 2). DNA from strain WM1-1 was used as the template to amplify upstream and downstream sequences of gene of interest, while plasmids pUC4K and pAX1.Cm were used as the templates for Km and Cm cassettes, respectively. Primers Up_R and Dn_F were 5′ extended with at least 21 bp of homology to the antibiotic cassette, thereby facilitating fusion of the upstream and downstream genomic fragments to the antibiotic resistance cassettes by overlap extension PCR. To accomplish this fusion, the three separately amplified fragments (upstream, antibiotic, and downstream) were purified from agarose gel and mixed in equal proportions in an overlap extension PCR using the end primers (UP_F and Dn_R). The fusion product, which contained the antibiotic resistance cassette flanked by genomic sequences upstream and downstream of the target, was purified from gel and stored at −20°C before use or was used as the template for amplification with the internal primer pair int_F/int_R (Table 2). All of the primers (Table 2) were designed with Primer3 Primer Design Tool in Geneious software (Biomatters). PCR was performed with a standard protocol, using an iProof High-Fidelity PCR kit (Bio-Rad) in an S1000 thermal cycler (Bio-Rad). PCR products were gel purified using either a 5PRIME gel extract minikit or Freeze 'N Squeeze DNA gel extraction spin columns (Bio-Rad).

Natural transformation of *X. fastidiosa* strains with PCR template and confirmation of mutants. Transformation of *X. fastidiosa* cells was performed with the gel-purified or internal primer-amplified PCR templates, using a natural transformation protocol as previously described (10, 13). Briefly, recipient *X. fastidiosa* strain WM1-1 and TemeculaL cultures from PW plates were suspended in PD3 liquid medium and optical density at 600 nm (OD_{600}) was adjusted to 0.25 (~10^8 cells · ml^{-1}). Ten μl of this suspension was spotted onto PD3 agar plates, 10 μl of PCR template was added on top of the spots, and the spots were dried before incubation at 28°C for 3 days. After 3 days, spots were suspended in 0.5 ml of PD3. Aliquots (100 μl) were spread plated onto PW plates containing necessary antibiotics for selection. After 2 weeks of incubation at 28°C, mutant CFU were enumerated and restreaked onto new antibiotic PW plates. Control spots of the strain cells without the addition of PCR template were also included for each experiment. Double mutants were generated by using *pilA1* mutants as recipients with the gel purified PCR template of *pilA2* deletion (construct 1926). Ten colonies per strain per construct were restreaked onto new antibiotic plates. After confirming that the colonies were resistant to the corresponding antibiotic, one colony per strain for each construct was selected for further analysis. Confirmation of gene deletion was performed by PCR and sequencing. PCR was performed with three sets of primer pairs, namely, the out pairs (out_F/out_R) that target the genomic region outside the recombination region of the construct; antibiotic primers (antb_F/antb_R); and respective gene primers (pilA1_F/pilA1_R and PD1926_F/PD1926_R). For each PCR, wild-type strains were included as controls (Fig. S3). Sanger sequencing of the mutant at the deleted genomic region was performed using primers targeting the outside region, internal region, and antibiotic region. Information on all of the primers is presented in Table 2.

Growth curve and biofilm formation. Growth curve and biofilm formation of the mutant and wild-type strains were assessed in 96-well plates during 5 days, as previously described (10). Experiments were repeated three times independently, with one or three identical 96-well plates used per experiment. For each wild-type strain or mutant background at least six wells (repetitions) on each plate were used.

Twitching motility. Twitching motility assessment of wild-type and mutant strains was performed in PD3 plates and PD3 plates supplemented with 50% Chardonnay sap, as previously described (10). Twitching motility was observed for at least 10 transformant colonies for each mutant. Selected colonies were restreaked, and repeated observation of these colonies for twitching motility was performed.

Complementation of *pilA2* mutants. Complementation of *pilA2* mutants was performed with the PCR-amplified and gel-purified complementation template prepared from the fusion PCR of three fragments (upstream *pilA2* complementation template, *pilA2* template containing the *pilA2* ORF its promoter and terminator region, and downstream *pilA2* complementation template). The upstream *pilA2* complementation construct was amplified from NS1::Km WM1-1 (10) using the primer pair pilA2C_up_F and pilA2C_up_R (Table 2). These primers amplify the upstream flanking region of NS1 and the Km cassette in one fragment. The 950-bp *pilA2* fragment contained a 237-bp upstream promoter region, a

TABLE 2 PCR and qRT-PCR primers and probes used in this study

Primer name	Sequence (5′–3′)[a]	Purpose or description	Amplicon size(s) (bp)	Source
1924_up_F	GCGGCACCACGTATATCAATAAAA	Amplify upstream region of *pilA1* (PD1924)	981	This study
1924_up_R	GCAACACCTTCTTCACGAGGCAGACAGAGAATTCATGTGTGGGGGTTTA			
1924_dn_F	GAGATTTTGAGACACAACGTGGCTTATGAATACACACAGCAACACGATC	Amplify downstream region of *pilA1* (PD1924)	1,190	This study
1924_dn_R	TTGGAGAAGAGGCGTGTTAAAAAC			
1924_int_F	ATATACAGGGTGCTTGCTGATTGA	Amplify internal region of *pilA1* construct	2,952	This study
1924_int_R	GGATGGGTTTAGGGATGCTGATAA			
1924_out_F	AAACCACCACCGATAACAACAATC	Confirm *pilA1* mutants (primers target outside the *pilA1* construct)	3,509, 2,884[b]	This study
1924_out_R	AATAGCGTTGGTAAGAAATCCAGC			
1926_up_F	CATTTCACTTTGACTTCACCCGAA	Amplify upstream region of *pilA2* (PD1926)	1,032	This study
1926_up_R	TGCCCCGTATTCAGTGTCGCTGATTAATAGCGTTGGTAAGAAATCCAGC			
1926_dn_F	GCCTGGTGCTACGCCTGAATAAGTGAAACACGATTCATGGGTAAATGCTC	Amplify downstream region of *pilA2* (PD1926)	700	This study
1926_dn_R	TGAGCGTCAATTTTAGAGGATGGA			
1926_int_F	TCAGCAATACTCATACTGGCACTT	Amplify internal region of *pilA2* construct	2,773	This study
1926_int_R	AACGTGTGCTTGAATCTTCGAATT			
1926_out_F	ACAAGAGTGAGCCGTTACAACTAT	Confirm *pilA2* mutants (primers target outside the *pilA2* construct)	3,004, 2,352[b]	This study
1926_out_R	CTTTTCCAATGAGCAGTTATCGGG			
Kan_F	GTCTGCCTCGTGAAG	Amplify kanamycin cassette	1,204	This study
Kan_R	AAGCCACGTTGTGT			
Cm_F	AATCAGCGACACTGAATACGG	Amplify chloramphenicol cassette	1,119	This study
Cm_R	TCACTTATTCAGGCGTAGCAC			
pilA2C_up_F	CGCGCCCGTTATTAATCGAA	Amplify upstream fragment of *pilA2* complementation construct (contains upstream of NS1 region and kanamycin cassette)	1,957	This study
pilA2C_up_R2	ATATTGAAGGGTGCAATACAAAGCATCTAGTCTCAACCATCATCGATGAA			
pilA2C_dn_F2	GTTTCAAGAGAGAGAGCGTTCAACACGATGCTGTTAACCATTGTCATC	Amplify downstream fragment of *pilA2* complementation construct (this is same as NS1 downstream fragment)	799	This study
pilA2C_dn_R	TAACCTTGTCAGCGTAGATG			
pilA2_F	ACAATTCATCGATGATGGTTGAGACTAGATGCTTTGTATTGCACCCTTCAAT	Amplify *pilA2* coding region, 237 bp upstream, and 216 bp downstream to include promoter and terminator regions	950	This study
pilA2_R	GCCATTGATGACAATGGTTAACAGCATCGTTTCAAGAGAGAGAGCGTTCAAC			
pilA_F	AAACACCGGACTTGCCAACATCAC	Primer and probe to amplify fragment of *pilA1*	140	30
pilA_R	TGTTGCATGTCCACTGACCTCCAT			
pilA_P[c]	AAACCATCGCTTGGAATCGTAGCGTCGA			
PD1926_F	CACTCCTAACGCTATTGGACTAC	Primer and probe to amplify fragment of *pilA2*	117	32
PD1926_R	TTGACCTGACCATTACCAATCA			
PD1926_P[c]	TGGTGGACATCACAACTACTGGCG			
nuoA_F	AGACGCACGGATGAAGTTCGATGT	Reference gene for qRT-PCR		30
nuoA_R	ATTCCAGCGCTCCCTTCTTCCATA			
nuoA_P[c]	TTCATCGTGCCTTGGACTCAGGTGTT			
1691_up F	AGGCAACCTGACAGCGATAC	Amplify upstream region of 1691 construct	541	This study
1691_up_R2	GCAACACCTTCTTCACGAGGCAGACCGTTGATGTTCGAGAAGTGCG			
1691_Dn_F2	GAGATTTTGAGACACAACGTGGCTTTGCATCAACCCCAAAGCTGA	Amplify downstream region of 1691 construct	504	This study
1691_Dn R	AAGCGATCCAATGAAGGGCT			
1691_int_F	ACGGCCCTTCTCTAAGATTGC	Amplify internal region of 1691 construct	2,152, 987[b]	This study
1691_int_R	TGTGTGGTGCTTGCATATTCTG			
1695_up_F	CGATGGCCTGTTGATAGCGATA	Amplify upstream region of 1695 construct	1,027	This study
1695_up_R	CCGTATTCAGTGTCGCTGATTGTCACCACGTTTGAGGAGTTTG			
1695_Dn_F	GTGCTACGCCTGAATAAGTGAAACGGGGTGAATGGACATTAG	Amplify downstream region of 1695 construct	1,018	This study
1695_Dn_R	CAGATGGGGAGTGCTGCTTTA			
1695_int_F	AAAGACGAAATCCTGGAGCTGTAT	Amplify internal region of 1695 construct	2,640, 1,546[b]	This study
1695_int_R	TTGATACCAATTGGAAGACAACGC			

[a]Underlining indicates the 5′ extended region of the primer that is homologous to one of the antibiotic resistance cassettes to facilitate fusion of chromosomal sequence to a selectable marker by overlap extension PCR.

[b]Amplicon size in the wild-type strain without the insertion of antibiotic resistance gene.

[c]These probes were used for reverse transcription-quantitative PCR (qRT-PCR) and were labeled with 6-carboxyfluorescein (FAM) at the 5′ end and Black Hole 588 Quencher-1 (BHQ1) at the 3′ end.

pilA2 coding region, and a 216-bp downstream region. This fragment was amplified using primer pair pilA2_F and pilA2_R and using the wild-type WM1-1 DNA as the template. The downstream complementation product was also amplified from wild-type WM1-1 DNA using primers pilA2C_dn_F and pilA2C_dn_R and is the same as the downstream region of NS1. Primers pilA2C_up_R, pilA2F, pilA2R, and pilA2C_Dn_F had a 27-bp extension at their 5′ ends that was homologous to the adjoining fragment. The individual fragments were amplified, gel purified, and fused by extension PCR as described above. The PCR fragments were then used in a natural transformation assay with both the wild-type strains WM1-1 and TemeculaL and their *pilA2* mutants.

RNA preparation and quantitative PCR. Wild-type *X. fastidiosa* TemeculaL, WM1-1, or mutant cells cultured in PW plates were scraped and adjusted to an OD_{600} of 0.25 as previously mentioned. Three 15-ml plastic tubes containing 3 ml of media (PD3, PD3 + BSA [PD3 supplemented with 3 g · liter^{-1} BSA], and Sap [100% *Vitis vinifera* var. Chardonnay sap; see reference 13]) were supplemented with 120 μl of the OD_{600}-adjusted TemeculaL strain. Tubes were incubated at 28°C with shaking at 150 rpm for 3 days.

Cells were then precipitated by centrifugation of the tubes at 5,000 rpm for 5 min at 4°C. Pellet formed was suspended in 200 μl of DNA/RNA Shield (Zymo Research), and RNA was extracted using a Quick-RNA MiniPrep Plus kit (Zymo Research), using in-column DNase treatment following the manufacturers' protocol. RNA concentration was measured by a NanoDrop 2000 spectrophotometer (Thermo Scientific). cDNA was prepared using qScript cDNA SuperMix (Quantabio) with 170 ng of normalized RNA in 20 μl volume. Quantitative PCR was performed using 1 μl of cDNA in 20 μl volume containing 4 μl of 5× PerfeCTa multiplex quantitative PCR (qPCR) ToughMix (Quantabio), 0.4 μM forward and reverse primers, and 0.2 μM TaqMan probe (labeled with 5′ 6-carboxyfluorescein [FAM] and 3′ Black Hole 588 Quencher-1 [BHQ-1]). Reactions were performed using a CFX96 real-time system (Bio-Rad) with the following cycling parameters: 95°C for 1 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Fold change in expression of *pilA1* and *pilA2* was calculated by the threshold cycle ($2^{-\Delta\Delta CT}$) method (68) using expression of *nuoA* gene as the endogenous control (30). Primers and probes used for gene expression are included in Table 2. Three independent replications were used.

Electron microscopy. Pili of wild-type and mutant strains were observed under a transmission electron microscope, following a protocol previously described (34). Two-day old cultures were scraped from PW agar plates and suspended in 200 μl of distilled water. Cell suspension (10 μl) was pipetted on Formvar-coated grid and subsequently stained with 50 μl of phosphotungstic acid. After 2 min, the excess liquid on the grid was removed by a filter paper, and then the grids were observed on a Zeiss EM 10 transmission electron microscope (Carl Zeiss, Jena, Germany). Two independent experiments were performed.

Statistical analysis. Data from biofilm formation were analyzed in SAS 9.3 (SAS Institute, Inc., Cary, NC) using GLIMMIX procedure, and means were separated by the Tukey-Kramer method ($P < 0.05$). To compare gene expression data, differences in fold change were analyzed by one-sample *t* test (fold change $\neq 1$, $P < 0.05$).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AEM.01167-18.

SUPPLEMENTAL FILE 1, PDF file, 1.0 MB.

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REFERENCES

1. Chatterjee S, Almeida RP, Lindow S. 2008. Living in two worlds: the plant and insect lifestyles of *Xylella fastidiosa*. Annu Rev Phytopathol 46:243–271. https://doi.org/10.1146/annurev.phyto.45.062806.094342.
2. Hopkins DL, Purcell AH. 2002. *Xylella fastidiosa*: cause of Pierce's disease of grapevine and other emergent diseases. Plant Dis 86:1056–1066. https://doi.org/10.1094/PDIS.2002.86.10.1056.
3. Newman KL, Almeida RP, Purcell AH, Lindow SE. 2003. Use of a green fluorescent strain for analysis of *Xylella fastidiosa* colonization of *Vitis vinifera*. Appl Environ Microbiol 69:7319–7327. https://doi.org/10.1128/AEM.69.12.7319-7327.2003.
4. Roper MC, Greve LC, Warren JG, Labavitch JM, Kirkpatrick BC. 2007. *Xylella fastidiosa* requires polygalacturonase for colonization and pathogenicity in *Vitis vinifera* grapevines. Mol Plant Microbe Interact 20:411–419. https://doi.org/10.1094/MPMI-20-4-0411.
5. Saponari M, Loconsole G, Cornara D, Yokomi RK, De Stradis A, Boscia D, Bosco D, Martelli GP, Krugner R, Porcelli F. 2014. Infectivity and transmission of *Xylella fastidiosa* by *Philaenus spumarius* (Hemiptera: Aphrophoridae) in Apulia, Italy. J Econ Entomol 107:1316–1319. https://doi.org/10.1603/EC14142.
6. De La Fuente L, Burr TJ, Hoch HC. 2007. Mutations in type I and type IV pilus biosynthetic genes affect twitching motility rates in *Xylella fastidiosa*. J Bacteriol 189:7507–7510. https://doi.org/10.1128/JB.00934-07.
7. Li Y, Hao G, Galvani CD, Meng Y, De La Fuente L, Hoch H, Burr TJ. 2007. Type I and type IV pili of *Xylella fastidiosa* affect twitching motility, biofilm formation and cell–cell aggregation. Microbiology 153:719–726. https://doi.org/10.1099/mic.0.2006/002311-0.
8. Meng Y, Li Y, Galvani CD, Hao G, Turner JN, Burr TJ, Hoch HC. 2005. Upstream migration of *Xylella fastidiosa* via pilus-driven twitching motility. J Bacteriol 187:5560–5567. https://doi.org/10.1128/JB.187.16.5560-5567.2005.
9. Mattick JS. 2002. Type IV pili and twitching motility. Annu Rev Microbiol 56:289–314. https://doi.org/10.1146/annurev.micro.56.012302.160938.
10. Kandel PP, Almeida RPP, Cobine PA, De La Fuente L. 2017. Natural competence rates are variable among *Xylella fastidiosa* strains and homologous recombination occurs in vitro between subspecies *fastidiosa* and *multiplex*. Mol Plant Microbe Interact 30:589–600. https://doi.org/10.1094/MPMI-02-17-0053-R.
11. Oliver JE, Cobine PA, De La Fuente L. 2015. *Xylella fastidiosa* isolates from both subsp. *multiplex* and *fastidiosa* cause disease on southern highbush blueberry (*Vaccinium* sp.) under greenhouse conditions. Phytopathology 105:855–862. https://doi.org/10.1094/PHYTO-11-14-0322-FI.
12. Oliver JE, Sefick SA, Parker JK, Arnold T, Cobine PA, De La Fuente L. 2014. Ionome changes in *Xylella fastidiosa*-infected *Nicotiana tabacum* correlate with virulence and discriminate between subspecies of bacterial isolates. Mol Plant Microbe Interact 27:1048–1058. https://doi.org/10.1094/MPMI-05-14-0151-R.
13. Kandel PP, Lopez SM, Almeida RP, De La Fuente L. 2016. Natural competence of *Xylella fastidiosa* occurs at a high frequency inside microfluidic chambers mimicking the bacterium's natural habitats. Appl Environ Microbiol 82:5269–5277. https://doi.org/10.1128/AEM.01412-16.
14. Guilhabert MR, Kirkpatrick BC. 2005. Identification of *Xylella fastidiosa* antivirulence genes: hemagglutinin adhesins contribute to *X. fastidiosa* biofilm maturation and colonization and attenuate virulence. Mol Plant Microbe Interact 18:856–868. https://doi.org/10.1094/MPMI-18-0856.
15. Newman KL, Almeida RP, Purcell AH, Lindow SE. 2004. Cell-cell signaling controls *Xylella fastidiosa* interactions with both insects and

plants. Proc Narl Acad Sci U S A 101:1737–1742. https://doi.org/10.1073/pnas.0308399100.
16. Wang N, Li J-L, Lindow SE. 2012. RpfF-dependent regulon of *Xylella fastidiosa*. Phytopathology 102:1045–1053. https://doi.org/10.1094/PHYTO-07-12-0146-R.
17. Burrows LL. 2012. *Pseudomonas aeruginosa* twitching motility: type IV pili in action. Annu Rev Microbiol 66:493–520. https://doi.org/10.1146/annurev-micro-092611-150055.
18. Morand PC, Drab M, Rajalingam K, Nassif X, Meyer TF. 2009. *Neisseria meningitidis* differentially controls host cell motility through PilC1 and PilC2 components of type IV Pili. PLoS One 4:e6834. https://doi.org/10.1371/journal.pone.0006834.
19. Wolfgang M, Park H-S, Hayes SF, Van Putten JP, Koomey M. 1998. Suppression of an absolute defect in type IV pilus biogenesis by loss-of-function mutations in *pilT*, a twitching motility gene in *Neisseria gonorrhoeae*. Proc Natl Acad Sci U S A 95:14973–14978.
20. Liu H, Kang Y, Genin S, Schell MA, Denny TP. 2001. Twitching motility of *Ralstonia solanacearum* requires a type IV pilus system. Microbiology 147:3215–3229. https://doi.org/10.1099/00221287-147-12-3215.
21. Bahar O, Goffer T, Burdman S. 2009. Type IV pili are required for virulence, twitching motility, and biofilm formation of *Acidovorax avenae* subsp. *citrulli*. Mol Plant Microbe Interact 22:909–920. https://doi.org/10.1094/MPMI-22-8-0909.
22. Dunger G, Guzzo CR, Andrade MO, Jones JB, Farah CS. 2014. *Xanthomonas citri subsp. citri* type iv pilus is required for twitching motility, biofilm development, and adherence. Mol Plant Microbe Interact 27:1132–1147. https://doi.org/10.1094/MPMI-06-14-0184-R.
23. Taguchi F, Ichinose Y. 2011. Role of type IV pili in virulence of *Pseudomonas syringae* pv. tabaci 6605: correlation of motility, multidrug resistance, and HR-inducing activity on a nonhost plant. Mol Plant Microbe Interact 24:1001–1011. https://doi.org/10.1094/MPMI-02-11-0026.
24. Burdman S, Bahar O, Parker JK, De La Fuente L. 2011. Involvement of type IV pili in pathogenicity of plant pathogenic bacteria. Genes 2:706–735. https://doi.org/10.3390/genes2040706.
25. Craig L, Pique ME, Tainer JA. 2004. Type IV pilus structure and bacterial pathogenicity. Nat Rev Microbiol 2:363–378. https://doi.org/10.1038/nrmicro885.
26. Seitz P, Blokesch M. 2013. Cues and regulatory pathways involved in natural competence and transformation in pathogenic and environmental Gram-negative bacteria. FEMS Microbiol Rev 37:336–363. https://doi.org/10.1111/j.1574-6976.2012.00353.x.
27. Simpson AJG, Reinach F, Arruda P, Abreu F, Acencio M, Alvarenga R, Alves LC, Araya J, Baia G, Baptista C. 2000. The genome sequence of the plant pathogen *Xylella fastidiosa*. Nature 406:151–157. https://doi.org/10.1038/35018003.
28. Van Sluys MA, de Oliveira MC, Monteiro-Vitorello CB, Miyaki CY, Furlan LR, Camargo LE, da Silva AC, Moon DH, Takita MA, Lemos EG, Machado MA, Ferro MI, da Silva FR, Goldman MH, Goldman GH, Lemos MV, El-Dorry H, Tsai SM, Carrer H, Carraro DM, de Oliveira RC, Nunes LR, Siqueira WJ, Coutinho LL, Kimura ET, Ferro ES, Harakava R, Kuramae EE, Marino CL, Giglioti E, Abreu IL, Alves LM, do Amaral AM, Baia GS, Blanco SR, Brito MS, Cannavan FS, Celestino AV, da Cunha AF, Fenille RC, Ferro JA, Formighieri EF, Kishi LT, Leoni SG, Oliveira AR, Rosa VE, Jr, Sassaki FT, Sena JA, de Souza AA, Truffi D, Tsukumo F, Yanai GM, Zaros LG, Civerolo EL, Simpson AJ, Almeida NF, Jr, Setubal JC, Kitajima JP. 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. https://doi.org/10.1128/JB.185.3.1018-1026.2003.
29. Cursino L, Galvani CD, Athinuwat D, Zaini PA, Li Y, De La Fuente L, Hoch HC, Burr TJ, Mowery P. 2011. Identification of an operon, Pil-Chp, that controls twitching motility and virulence in *Xylella fastidiosa*. Mol Plant Microbe Interact 24:1198–1206. https://doi.org/10.1094/MPMI-10-10-0252.
30. Cruz LF, Parker JK, Cobine PA, De La Fuente L. 2014. Calcium-enhanced twitching motility in *Xylella fastidiosa* is linked to a single PilY1 homolog. Appl Environ Microbiol 80:7176–7185. https://doi.org/10.1128/AEM.02153-14.
31. da Silva Neto JF, Koide T, Abe CM, Gomes SL, Marques MV. 2008. Role of σ54 in the regulation of genes involved in type I and type IV pili biogenesis in *Xylella fastidiosa*. Arch Microbiol 189:249–261. https://doi.org/10.1007/s00203-007-0314-x.
32. Parker JK, Chen H, McCarty SE, Liu LY, De La Fuente L. 2016. Calcium transcriptionally regulates the biofilm machinery of *Xylella fastidiosa* to promote continued biofilm development in batch cultures. Environ Microbiol 18:1620–1634. https://doi.org/10.1111/1462-2920.13242.
33. Matsumoto A, Young GM, Igo MM. 2009. Chromosome-based genetic complementation system for *Xylella fastidiosa*. Appl Environ Microbiol 75:1679–1687. https://doi.org/10.1128/AEM.00024-09.
34. Chen H, Kandel PP, Cruz LF, Cobine PA, De La Fuente L. 2017. The major outer membrane protein MopB is required for twitching movement and affects biofilm formation and virulence in two *Xylella fastidiosa* strains. Mol Plant Microbe Interact 30:896–905. https://doi.org/10.1094/MPMI-07-17-0161-R.
35. Kung SH, Almeida RP. 2014. Biological and genetic factors regulating natural competence in a bacterial plant pathogen. Microbiology 160:37–46. https://doi.org/10.1099/mic.0.070581-0.
36. Davis MJ, French WJ, Schaad NW. 1981. Axenic culture of the bacteria associated with phony disease of peach and plum leaf scald. Curr Microbiol 6:309–314. https://doi.org/10.1007/BF01566883.
37. Davis M, Purcell A, Thomson S. 1980. Isolation media for the Pierce's disease bacterium. Phytopathology 70:425–429. https://doi.org/10.1094/Phyto-70-425.
38. Killiny N, Almeida RPP. 2009. Host structural carbohydrate induces vector transmission of a bacterial plant pathogen. Proc Natl Acad Sci U S A 106:22416–22420. https://doi.org/10.1073/pnas.0908562106.
39. Killiny N, Almeida RP. 2009. *Xylella fastidiosa* afimbrial adhesins mediate cell transmission to plants by leafhopper vectors. Appl Environ Microbiol 75:521–528. https://doi.org/10.1128/AEM.01921-08.
40. Killiny N, Prado SS, Almeida RP. 2010. Chitin utilization by the insect-transmitted bacterium *Xylella fastidiosa*. Appl Environ Microbiol 76:6134–6140. https://doi.org/10.1128/AEM.01036-10.
41. Hao L, Athinuwat D, Johnson K, Cursino L, Burr T, Mowery P. 2017. *Xylella fastidiosa pil-chp* operon is involved in regulating key structural genes of both type I and IV pili. VITIS 56:55–62.
42. Kazmierczak BI, Hendrixson DR. 2013. Spatial and numerical regulation of flagellar biosynthesis in polarly flagellated bacteria. Mol Microbiol 88:655–663. https://doi.org/10.1111/mmi.12221.
43. Viollier PH, Sternheim N, Shapiro L. 2002. A dynamically localized histidine kinase controls the asymmetric distribution of polar pili proteins. EMBO J 21:4420–4428. https://doi.org/10.1093/emboj/cdf454.
44. Viollier PH, Sternheim N, Shapiro L. 2002. Identification of a localization factor for the polar positioning of bacterial structural and regulatory proteins. Proc Natl Acad Sci U S A 99:13831–13836. https://doi.org/10.1073/pnas.182411999.
45. Cowles KN, Moser TS, Siryaporn A, Nyakudarika N, Dixon W, Turner JJ, Gitai Z. 2013. The putative Poc complex controls two distinct *Pseudomonas aeruginosa* polar motility mechanisms. Mol Microbiol 90:923–938. https://doi.org/10.1111/mmi.12403.
46. de Souza AA, Takita MA, Coletta-Filho HD, Caldana C, Goldman GH, Yanai GM, Muto NH, de Oliveira RC, Nunes LR, Machado MA. 2003. Analysis of gene expression in two growth states of *Xylella fastidiosa* and its relationship with pathogenicity. Mol Plant Microbe Interact 16:867–875. https://doi.org/10.1094/MPMI.2003.16.10.867.
47. de Souza AA, Takita MA, Pereira EO, Coletta-Filho HD, Machado MA. 2005. Expression of pathogenicity-related genes of *Xylella fastidiosa in vitro* and *in planta*. Curr Microbiol 50:223–228. https://doi.org/10.1007/s00284-004-4447-8.
48. Caserta R, Takita M, Targon M, Rosselli-Murai L, De Souza A, Peroni L, Stach-Machado D, Andrade A, Labate C, Kitajima E. 2010. Expression of *Xylella fastidiosa* fimbrial and afimbrial proteins during biofilm formation. Appl Environ Microbiol 76:4250–4259. https://doi.org/10.1128/AEM.02114-09.
49. Ishimoto KS, Lory S. 1989. Formation of pilin in *Pseudomonas aeruginosa* requires the alternative sigma factor (RpoN) of RNA polymerase. Proc Natl Acad Sci U S A 86:1954–1957.
50. Totten PA, Lara JC, Lory S. 1990. The *rpoN* gene product of *Pseudomonas aeruginosa* is required for expression of diverse genes, including the flagellin gene. J Bacteriol 172:389–396. https://doi.org/10.1128/jb.172.1.389-396.1990.
51. Kehl-Fie TE, Porsch EA, Miller SE, StGeme JW. 2009. Expression of *Kingella kingae* type IV pili is regulated by σ54, PilS, and PilR. J Bacteriol 191:4976–4986. https://doi.org/10.1128/JB.00123-09.
52. Parker D, Kennan RM, Myers GS, Paulsen IT, Songer JG, Rood JI. 2006. Regulation of type IV fimbrial biogenesis in *Dichelobacter nodosus*. J Bacteriol 188:4801–4811. https://doi.org/10.1128/JB.00255-06.
53. Ray SK, Kumar R, Peeters N, Boucher C, Genin S. 2015. *rpoN1*, but not *rpoN2*, is required for twitching motility, natural competence, growth on

nitrate, and virulence of *Ralstonia solanacearum*. Front Microbiol 6:229. https://doi.org/10.3389/fmicb.2015.00229.
54. Bretl DJ, Müller S, Ladd KM, Atkinson SN, Kirby JR. 2016. Type IV-pili dependent motility is co-regulated by PilSR and PilS2R2 two-component systems via distinct pathways in *Myxococcus xanthus*. Mol Microbiol 102:37–53. https://doi.org/10.1111/mmi.13445.
55. Francke C, Groot Kormelink T, Hagemeijer Y, Overmars L, Sluijter V, Moezelaar R, Siezen RJ. 2011. Comparative analyses imply that the enigmatic sigma factor 54 is a central controller of the bacterial exterior. BMC Genomics 12:385. https://doi.org/10.1186/1471-2164-12-385.
56. Zhang N, Buck M. 2015. A perspective on the enhancer dependent bacterial RNA polymerase. Biomolecules 5:1012–1019. https://doi.org/10.3390/biom5021012.
57. Zhao K, Liu M, Burgess RR. 2010. Promoter and regulon analysis of nitrogen assimilation factor, σ54, reveal alternative strategy for *E. coli* MG1655 flagellar biosynthesis. Nucleic Acids Res 38:1273–1283. https://doi.org/10.1093/nar/gkp1123.
58. Galvani CD, Li Y, Burr TJ, Hoch HC. 2007. Twitching motility among pathogenic *Xylella fastidiosa* isolates and the influence of bovine serum albumin on twitching-dependent colony fringe morphology. FEMS Microbiol Lett 268:202–208.
59. da Silva Neto JF, Koide T, Gomes SL, Marques MV. 2002. Site-directed gene disruption in *Xylella fastidiosa*. FEMS Microbiol Lett 210:105–110.
60. Monteiro PcB, Teixeira DC, Palma RR, Garnier M, Bové J-M, Renaudin J. 2001. Stable transformation of the *Xylella fastidiosa* citrus variegated chlorosis strain with *oriC* plasmids. Appl Environ Microbiol 67:2263–2269. https://doi.org/10.1128/AEM.67.5.2263-2269.2001.
61. Navarrete F, De La Fuente L. 2015. Zinc detoxification is required for full virulence and modification of the host leaf ionome by *Xylella fastidiosa*. Mol Plant Microbe Interact 28:497–507. https://doi.org/10.1094/MPMI-07-14-0221-R.
62. Wells JM, Raju BC, Hung HY, Weisburg WG, Mandelco-Paul L, Brenner DJ. 1987. *Xylella fastidiosa* gen. nov., sp. nov.: Gram-negative, xylem-limited, fastidious plant bacteria related to *Xanthomonas* spp. Int J Syst Bacteriol 37:136–143. https://doi.org/10.1099/00207713-37-2-136.
63. Kung SH, Almeida RP. 2011. Natural competence and recombination in the plant pathogen *Xylella fastidiosa*. Appl Environ Microbiol 77:5278–5284. https://doi.org/10.1128/AEM.00730-11.
64. Gomaa AE, Deng Z, Yang Z, Shang L, Zhan Y, Lu W, Lin M, Yan Y. 2017. High-frequency targeted mutagenesis in *Pseudomonas stutzeri* using a vector-free allele-exchange protocol. J Microbiol Biotechnol 27:335–341. https://doi.org/10.4014/jmb.1608.08019.
65. Aranda J, Poza M, Pardo BG, Rumbo S, Rumbo C, Parreira JR, Rodríguez-Velo P, Bou G. 2010. A rapid and simple method for constructing stable mutants of *Acinetobacter baumannii*. BMC Microbiol 10:279. https://doi.org/10.1186/1471-2180-10-279.
66. Zhang S, Zou ZZ, Kreth J, Merritt J. 2017. Recombineering in *Streptococcus mutans* using direct repeat-mediated cloning-independent markerless mutagenesis (DR-CIMM). Front Cell Infect Microbiol 7:202. https://doi.org/10.3389/fcimb.2017.00202.
67. Parker JK, Havird JC, De La Fuente L. 2012. Differentiation of *Xylella fastidiosa* strains via multilocus sequence analysis of environmentally mediated genes (MLSA-E). Appl Environ Microbiol 78:1385–1396. https://doi.org/10.1128/AEM.06679-11.
68. Schmittgen TD, Livak KJ. 2008. Analyzing real-time PCR data by the comparative CT method. Nat Protoc 3:1101–1108. https://doi.org/10.1038/nprot.2008.73.