The Major Outer Membrane Protein MopB Is Required for Twitching Movement and Affects Biofilm Formation and Virulence in Two *Xylella fastidiosa* strains

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MopB is a major outer membrane protein (OMP) in *Xylella fastidiosa*, a bacterial plant pathogen that causes losses on many economically important crops. Based on in silico analysis, the uncharacterized MopB protein of *X. fastidiosa* contains a β -barrel structure with an OmpA-like domain and a predicted calcium-binding motif. Here, MopB function was studied by mutational analysis taking advantage of the natural competence of *X. fastidiosa*. Mutants of *mopB* were constructed in two different *X. fastidiosa* strains, the type strain Temecula and the more virulent WM1-1. Deletion of the *mopB* gene impaired cell-to-cell aggregation, surface attachment, and biofilm formation in both strains. Interestingly, *mopB* deletion completely abolished twitching motility. Electron microscopy of the bacterial cell surface revealed that *mopB* deletion eliminated type IV and type I pili formation, potentially caused by destabilization of the outer membrane. Both *mopB* mutants showed reduced virulence using tobacco (*Nicotiana tabacum*) as a host under greenhouse conditions. These results suggest that MopB has pleiotropic functions in biofilm formation and twitching motility and is important for virulence of *X. fastidiosa*.

Xylella fastidiosa is a gram-negative plant-pathogenic bacterium that causes many economically important diseases, including Pierce's disease (PD) in grapevine and citrus variegated chlorosis (Almeida and Nunney 2015; Bae et al. 2015). The pathogen is transmitted by xylem sap-feeding insects such as sharpshooters, leafhoppers, and spittlebugs, by directly injecting the bacterium into xylem vessels (Almeida et al. 2003; Chatterjee et al. 2008). The bacterium strictly colonizes the xylem vessels (Chatterjee et al. 2008) and, therefore, is difficult to control, due to the challenge of common antibacterial treatments reaching the pathogen. Currently there are no effective long-term practices to control the devastating diseases caused by *X. fastidiosa*.

The virulence mechanisms of *X. fastidiosa* are not entirely understood. The current working hypothesis is that disease symptoms caused by *X. fastidiosa* infection are the result of long term water deficit, which is associated with the blockage of xylem vessels by bacterial biofilms and plant-produced gels and tyloses (Chatterjee et al. 2008; Sun et al. 2013). Biofilm formed by different pathogens is vital for its persistence in a nutrient-poor plant xylem system (Castiblanco and Sundin 2015). Additionally, for systemic colonization of the plant xylem system, upstream migration is important, which is mediated by type IV pilus-driven twitching motility (Meng et al. 2005). Therefore, twitching motility and biofilm formation are critical virulence traits of *X. fastidiosa*. Previous studies showed that two regulatory genes, *algU* and *xhpT*, which encode proteins with roles in environmental stress response and regulation of cell-cell aggregation and biofilm formation, are important for virulence (Shi et al. 2007; Voegel et al. 2013). Other factors implicated in virulence include secreted proteins (Nascimento et al. 2016; Zhang et al. 2015), such as a functional polygalacturonase encoded by *pglA* (Roper et al. 2007) and a lipase encoded by *lesA* (Nascimento et al. 2016).

Previous studies from our group indicated that mineral elements in the xylem, such as zinc (Zn) and calcium (Ca), can affect traits associated with virulence of *X. fastidiosa*. Zn was shown to be a stress factor with pleiotropic effects on *X. fastidiosa*, decreasing growth and biofilm formation of the bacterium but increasing exopolysaccharide production and adhesion strength of biofilms (Navarrete and De La Fuente 2014). Ca was found to increase cell attachment, biofilm formation, and twitching motility of *X. fastidiosa* (Cruz et al. 2012). Ca was also found to accumulate at higher concentrations in biofilm than in planktonic cells (Cobine et al. 2013). Ca is a divalent cation that can function as an ion bridge between bacterial cells as well as between bacterial cells and xylem surfaces (Leite et al. 2002). However, previous research by our group found that disrupting de novo protein synthesis abolishes the Ca-induced increase in surface attachment by *X. fastidiosa*. (Cruz et al. 2012). This research suggested a regulatory effect of Ca on *X. fastidiosa* virulence traits. Ca also interacts with a particular homolog of PilY1, a type IV pilus structural protein that contains a Ca-binding motif, enhancing twitching motility of *X. fastidiosa* (Cruz et al. 2014). Recently, whole transcriptome analyses conducted by our group indicated that gene expression of biofilm cells is broadly modified by Ca supplementation (Parker et al. 2016). These results suggest multiple regulatory roles for Ca in *X. fastidiosa*.

Proteins in the outer membrane of gram-negative bacteria have many functions, such as serving as transporters, recognizing

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proteins and components of signal transduction cascades, and maintaining integrity of this structure (Koebnik et al. 2000). In *X. fastidiosa*, the outer membrane protein (OMP) MopB (PD1709) has been used as a recognition target in an engineered antimicrobial therapeutics approach for protection against *X. fastidiosa* (Dandekar et al. 2012). MopB is required for pathogenicity in *Xanthomonas campestris* pv. *campestris*, the causal agent of black rot in crucifers (Chen et al. 2010), but its role in virulence of *X. fastidiosa* has not been studied.

In this study, *mopB* mutants in two *X. fastidiosa* wild-type (WT) strains, Temecula and WM1-1, were constructed by site-directed mutagenesis. Temecula is the type strain for PD in grapevine, and its whole genome has been sequenced (Van Sluys et al. 2003). WM1-1 is an aggressive strain that our research group isolated from naturally-infected grapevines in Georgia (Oliver et al. 2014; Parker et al. 2012). The response of the mutants to Ca supplementation and its effect on adhesiveness, biofilm formation, twitching motility, and disease development in planta were evaluated.

RESULTS

In silico analysis of the MopB homolog (PD1709) of *X. fastidiosa*.

The 1,170-bp open reading frame of PD1709 in the *X. fastidiosa* Temecula genome was identified as the homolog of *Xanthomonas campestris* pv. *campestris* MopB protein (100% coverage and 70% similarity). PD1709 was the only MopB homolog found in the Temecula genome and the other *X. fastidiosa* genomes available at the National Center for Biotechnology Information (NCBI). The N-terminal domain of MopB forms an antiparallel β barrel, which is predicted to be embedded in the outer membrane, and the C-terminal half has an OmpA domain located in the periplasmic space. This predicted structure is very similar to proteins in the OmpA family (Supplementary Fig. S1). In addition, a putative EF-hand motif, Dx[DN]x[DE], is located in the C-terminal half of the protein (Parker et al. 2016).

Generation of *mopB* knockout mutants of *X. fastidiosa* Temecula and WM1-1.

Taking advantage of natural competence of *X. fastidiosa*, the vector pJET_mopB_Kan was introduced into *X. fastidiosa* Temecula and WM1-1, generating *mopB* knockout mutants in both strains. Mutation was confirmed by the absence of the *mopB* gene and insertion of a kanamycin-resistance cassette in the *mopB* locus (Supplementary Fig. S2A). In addition,

sequencing of the regions upstream and downstream of *mopB* confirmed that insertion of the kanamycin cassette did not modify adjacent genes (data not shown).

Crude extracts of proteins of WT strains and *mopB* mutants were separated by sodium dodecyl sulfate-polyacrylamide (SDS-PAGE) gel electrophoresis. A major protein band of approximately 42 kDa was detected in WT strains but not in *mopB* mutants. This major protein band was excised from the gel, was digested, and was subjected to identification by liquid chromatography-tandem mass spectrometry (LC-MS/MS). The top hit in the analysis (using the Global Proteome Machine) was the MopB homolog in *X. fastidiosa* 9a5c (due to available sequences in database), with 10 peptides identified with 14% coverage out of a maximum expected coverage of 19% and a log(e) of -27.8 that reflects the probability of randomly assigning this protein with this dataset. Therefore, the major band was *X. fastidiosa* MopB.

Effect of *mopB* mutation on planktonic growth and biofilm formation of *X. fastidiosa*.

To characterize *mopB* mutants of *X. fastidiosa*, the effect of these mutations on planktonic growth and biofilm formation was evaluated. When grown in PD2 broth, similar planktonic growth was observed between the *mopB* mutant and its isogenic WT strain (Fig. 1A). Similar results were observed when all the strains were grown in 4 mM Ca-supplemented PD2 broth (4Ca) (Fig. 1B).

In 96-well plate assays, biofilm formation of each strain was quantified at 7 days postinoculation (dpi). In all the culture media tested (PD2, 4Ca, 50% 'Chardonnay' xylem fluid [50% Sap], and 'Chardonnay' xylem fluid [Sap]), biofilm formed by *mopB* mutants was significantly less ($P < 0.05$) than the corresponding WT strains (Fig. 1C). In the three media containing higher Ca concentration than PD2, biofilm growth of each strain was significantly increased ($P < 0.05$).

MopB is important for cell aggregation and surface attachment.

Surface attachment and cell aggregation are initial stages in the process of biofilm formation (Castiblanco and Sundin 2015). The strength of surface attachment was assessed by measuring the percentage of attached cells and calculating the adhesion force for Temecula and *mopB*(T), using microfluidic chambers. When the flow rate was higher than $40 \mu\text{l min}^{-1}$, the percentage of attached *mopB*(T) cells decreased dramatically when compared with WT Temecula cells (Fig. 2A). A significant reduction in adhesion force ($P = 0.02$) was observed for *mopB*(T)

Fig. 1. Planktonic growth and biofilm formation of *Xylella fastidiosa* strains under different Ca concentration. **A** and **B**, Planktonic growth curve of *X. fastidiosa* wild type (WT) strains and *mopB* mutants in 96-well plates containing PD2 broth (PD2) and PD2 broth supplemented with 4 mM CaCl_2 (4Ca) for 7 days. **C**, Biofilm quantification of WT strains and *mopB* mutants cultured in 96-well plates for 7 days. PD2, 4Ca, 50% Sap, and Sap represent PD2 broth, PD2 broth supplemented with 4mM CaCl_2 , 50% 'Chardonnay' grape xylem fluid, and 100% 'Chardonnay' grape xylem fluid. Each data point represents means ($n = 12$) and standard error of the mean from one representative experiment. Three independent experiments were performed under the same conditions. Different letters indicate significant differences ($P < 0.05$) of biofilm formation among *X. fastidiosa* strains, according to the GLIMMIX procedure using SAS 9.4 (SAS Institute Inc.).

(139.4 ± 29.9 pN) compared with Temecula (221.8 ± 24.4 pN), as shown in Figure 2B.

Cell aggregation of Temecula and *mopB*(T) were observed in microfluidic chambers (Fig. 2C). At 5 dpi, aggregates of Temecula cells were observed in several sections of the microchannel. In contrast, aggregates of *mopB*(T) cells were rarely observed and the size of the aggregates was much smaller than that of Temecula. Additionally, cell aggregation of the two WT strains and their corresponding mutants was quantified by calculating the relative percentage of aggregated cells in 50-ml conical tubes (Fig. 2D). Percentage of aggregated cells of *mopB* mutants was significantly lower ($P < 0.001$) than that of the WT strains in PD2. However, in 4Ca, there was no significant difference between the WT and mutant strains.

mopB mutants are nonmotile.

Colony fringe width on agar plates is a parameter used to measure twitching motility of *X. fastidiosa*. WT strains and *mopB* mutants on periwinkle wilt (PW) and PW agar plates supplemented with 2 mM Ca were used to measure colony fringe. In all conditions, peripheral fringe was detected for WT Temecula and WM1-1 (Fig. 3A) but the edge of *mopB* mutant colonies was smooth and there was no visible peripheral fringe (Fig. 3A). On the PW plate, the width of the fringe in WM1-1 (mean = 49.78 ± 1.78 μm) was significantly higher than that of Temecula (mean = 26.71 ± 1.02 μm) ($P < 0.001$) (Fig. 3B). In 2 mM Ca-supplemented PW plates, fringe width of both WT strains was increased (Fig. 3C) and the difference between WM1-1 (mean = 84.45 ± 2.63 μm) and Temecula (mean = 45.08 ± 2.62 μm) was still significant ($P < 0.001$).

In microfluidic chambers, the twitching movement of single cells was visible (Supplementary Video S1). Temecula cells inside channels of the microfluidic chambers not only moved with the medium flow but, also, moved against the flow direction. In contrast, the upstream movement of *mopB*(T) cells was not observed. The average twitching speed of Temecula cells was 0.45 μm min⁻¹ (Fig. 3D). Results obtained from microfluidic chamber experiments confirmed colony fringe observations. Transmission electron micrographs revealed the presence of pili on one pole of the WT *X. fastidiosa* strains (Fig. 4), as previously described (Meng et al. 2005). In contrast, no type I and type IV pili were observed in either of the *mopB* mutants (Fig. 4).

mopB mutation causes a decrease in virulence in both *X. fastidiosa* wild-type strains.

Previous reports showed that tobacco is a suitable model plant for studying virulence of *X. fastidiosa* and investigating *X. fastidiosa*-host interactions (De La Fuente et al. 2013; Francis et al. 2008; Oliver et al. 2014). 'Petite Havana SR1' tobacco plants inoculated with the WT *X. fastidiosa* strains showed symptoms including leaf scorch and leaf curling. The earliest symptoms were observed in the WT-inoculated plants approximately 70 to 75 dpi. One or two weeks later, symptoms were visible on the *mopB*(W)- and *mopB*(T)-inoculated plants (Fig. 5A). During monitoring of symptoms for 4 weeks, the disease incidence of plants inoculated with the WT strains was always higher than that of the plants inoculated with the *mopB* mutants (Fig. 5A). The mutation also affected severity of symptoms in leaves (Fig. 5B). At 90 to 95 dpi, leaf-scorch symptoms were

Fig. 2. Evaluation of cell aggregation and surface attachment of *Xylella fastidiosa* wild type (WT) and *mopB* mutants. **A**, Cell surface attachment inside microfluidic chambers as a function of increased medium flow rate. The data represented in the graph corresponds to one representative experiment. Three independent experiments were performed under the same conditions. **B**, Adhesion force, measured in piconewtons (pN), of *X. fastidiosa* Temecula and *mopB*(T) was assessed in microfluidic chambers. Adhesion force refers the amount of force necessary for cell detachment. Data presented was obtained from three independent experiments. Error bars represent standard errors of the mean ($n = 3$). Different letters on top of the bars indicate significant differences ($P < 0.05$) according to a Student's *t* test. **C**, Time-lapse micrographs showing formation of cell aggregates inside microfluidic chambers. Images were captured at 1 and 5 days postinoculation (dpi). **D**, Percentage of aggregated cells grown in 50-ml conical tubes. Each data point represents means ($n = 3$) and standard error of the mean from one representative experiment. Three independent experiments were performed under the same conditions. Different letters on top of the bars indicate significant differences ($P < 0.05$), according to the GLIMMIX procedure using SAS 9.4 (SAS Institute Inc.).

40% fewer in *mopB*(T) mutant-inoculated plants, as compared with Temecula-inoculated plants, and 53% fewer in *mopB*(W) mutant-inoculated plants, as compared with WM1-1-inoculated plants (Fig. 5C).

Colonization of *X. fastidiosa* in tobacco plants.

Quantitative polymerase chain reaction (qPCR) detection indicated 100% of the infected plants were colonized by *X. fastidiosa*. There was no significant difference in bacterial populations in leaf petioles of plants infected by WT strains and *mopB* mutants at 90 to 95 dpi. For plants infected with Temecula and *mopB*(T), the average bacterial population detected in infected plant leaf petioles was 3.72 ± 0.22 and 3.84 ± 0.26 log CFU mg⁻¹, respectively. For plants infected with WM1-1 and *mopB*(W), the average bacterial population detected was 3.62 ± 0.11

and 3.78 ± 0.12 log CFU mg⁻¹, respectively. In addition, *X. fastidiosa* isolated from plants infected by WT strains grew on PW plates but did not grow on PW plates with 50 µg of kanamycin per milliliter, confirming the lack of kanamycin resistance. *X. fastidiosa* isolated from plants infected by mutants not only grew on PW plates but also grew on PW plates with 50 µg of kanamycin per milliliter, confirming that these infecting isolates retained the kanamycin resistance cassette inserted in the *mopB* locus.

DISCUSSION

In this study, we have shown that the major OMP MopB has a pleiotropic effect that influences virulence in *X. fastidiosa*. Using two strains of *X. fastidiosa*, deletion of *mopB* impaired

Fig. 3. Twitching motility of *Xylella fastidiosa* wild-type (WT) strains and *mopB* mutants. **A**, Representative micrographs of bacteria colony fringe on periwinkle wilt (PW) plates. **B** and **C**, Colony fringe width of *X. fastidiosa* WT strains and *mopB* mutants on PW plates and PW plates supplemented with 2 mM CaCl₂. Each data point represents means ($n = 12$) and standard error (SE) of the mean from one representative experiment. Four independent experiments were performed under the same conditions. Different letters on top of the bars indicate significant differences ($P < 0.05$), according to the GLIMMIX procedure using SAS 9.4 (SAS Institute Inc.). n.d. = not detected. **D**, Twitching speed assessments of *X. fastidiosa* Temecula and *mopB*(T) in microfluidic chambers. Different letters on top of the bars indicate significant differences ($P < 0.05$) according to a Student's *t* test. Data presented was obtained from three independent experiments. Error bars correspond to SE of the mean ($n = 9$).

Fig. 4. Transmission electron micrographs of negatively stained cells of *Xylella fastidiosa* wild type (WT) strains and *mopB* mutants. White arrows indicate type I pili and black arrows indicate type IV pili on the polar end of WT strains. No pili were observed in any of the *mopB* mutants. The magnification is 10,000 $\times$, images were captured on a Zeiss EM 10 transmission electron microscope. Scale bar on bottom left indicates 1 µm.

surface attachment and biofilm formation, abolished twitching motility, and attenuated disease development. Our findings show, for the first time, that MopB is required for bacterial type IV pili biogenesis and twitching motility and contribute to our understanding of the requirements for virulence in *X. fastidiosa*.

In a previous study conducted by our group, Ca increased surface attachment, biofilm formation, and twitching motility of *X. fastidiosa* (Cruz et al. 2012). Additionally, in other bacterial species, several studies showed that Ca-binding by proteins containing conserved Ca-binding motifs help regulate biofilm formation (Arrizubieta et al. 2004; Sarkisova et al. 2014). The sequence motif Dx[DN]xDxxxxx[DE], known as an EF-hand, was found in the C-terminus of *X. fastidiosa* MopB (Parker et al. 2016); therefore, we hypothesized that Ca-binding by the OMP MopB would have an important role in the response to Ca during biofilm formation by *X. fastidiosa*. We observed that *mopB* mutants were significantly impaired in biofilm formation under normal laboratory conditions, and, while biofilm formation could be enhanced in *mopB* mutants by addition of Ca, it was still significantly reduced compared with WT. Ca concentrations of approximately 4 mM in 4Ca media and Sap and 2 mM in 50% Sap are much higher than that of PD2 broth (approximately 0.02 mM) (Cobine et al. 2013), suggesting that *mopB* mutants may still be able to form biofilm in planta.

Biofilm formed by WT strains and the *mopB* mutants was increased under higher Ca concentration conditions. These results suggest that MopB is probably not the key protein responsible for Ca-induced increase in biofilm formation by *X. fastidiosa*. Interestingly, the effect of *mopB* deletion on reduced cell aggregation was compensated by adding Ca to the medium. In PD2 broth, the percentage of aggregated *mopB* mutant cells was less than that of WT strains, but 4 mM Ca eliminated this difference. This suggests that Ca may be acting as an “ionic glue” at the surface, interacting with other surface proteins besides MopB. Ca is a divalent cation that can function as an ion bridge between negatively charged surfaces (such as bacteria) and therefore contributes to bacterial adhesion (Leite et al. 2002). Recently, it has been reported that Ca binding to extracellular DNA mediates bacterial aggregation in a variety of bacterial strains (Das et al. 2014), which could be another explanation for the observed effect.

Although MopB is not required for Ca-induced increase in biofilm formation, this protein was shown here to be important for biofilm and disease development and fundamental for pili formation and, therefore, for twitching movement. Biofilm is important for *X. fastidiosa* inhabiting the xylem environment and the processes leading to disease development (Almeida et al. 2003; Chatterjee et al. 2008). Biofilm formation of this pathogen has been extensively studied, and a number of studies

Fig. 5. Virulence assessment of *Xylella fastidiosa* wild type (WT) strains and *mopB* mutants in tobacco plants in the greenhouse. **A**, Disease incidence in tobacco plant infected with *X. fastidiosa*. Symptom evaluation for each strain was performed during 4 weeks after the first leaf scorch symptoms were evident. Disease incidence is represented by the percentage of plants with symptoms among all eight inoculated plants. **B**, The mutation of *mopB* affected severity of symptoms in leaves. Photographs of tobacco plants were taken at 95 days postinoculation (dpi). **C**, Symptom quantification of plants inoculated with *X. fastidiosa* strains at 90 to 95 dpi. For each strain, the average percentage of symptomatic leaves showing marginal leaf scorch of an inoculated plant was represented in the graph. Each data point represents means ($n = 24$) and error bars correspond to standard error of the mean. Data were obtained from three independent experiments. Different letters on the bars indicate significant differences ($P < 0.05$), according to the GLIMMIX procedure using SAS 9.4 (SAS Institute Inc.).

indicated that surface attachment and cell aggregation are important in the early stages of biofilm development (Cruz et al. 2012; Guilhabert and Kirkpatrick 2005). Our findings show that *mopB* deletion decreases the strength of the bacterial attachment to a surface and the percentage of aggregated cells, concomitantly affecting biofilm formation. OMPs are known to be important in biofilm formation, as demonstrated in previous studies using proteomic analysis, revealing that the major OMP OmpA is overexpressed during biofilm formation by *Escherichia coli* (Orme et al. 2006). A series of genetic studies showed that mutation of genes encoding OMPs affect biofilm production. In *E. coli*, deletion of *ompA* increases biofilm formation on hydrophobic surfaces and decreases biofilm formation on hydrophilic surfaces. This was shown to be related to cellulose synthesis, which responds to cell-surface stress and is regulated through the CpxRA stress response system (Ma and Wood 2009). OprF of *Pseudomonas aeruginosa* is homologous to OmpA of *E. coli*, deletion of *oprF* increased biofilm formation by *P. aeruginosa*. Absence of OprF caused cell-envelope stress that results in elevated levels of cyclic diguanylate monophosphate (c-di-GMP). The change of c-di-GMP in turn regulated Pel synthesis, affecting Pel-dependent phenotypes such as cell aggregation and biofilm formation (Bouffartigues et al. 2015). MopB has a similar structure to the OmpA family; therefore, MopB function may be similar to OmpA.

The phenotypic effects of *mopB* deletion may also be a consequence of cell-surface changes that directly or indirectly influence biofilm formation of *X. fastidiosa*. In this study, cell-surface structures, type I and type IV pili, were not observed at the pole of *mopB* mutant cells, using transmission electron microscopy. Transmission electron micrographs of one or both *X. fastidiosa* type I and IV pili-deficient strains have been presented in previous studies (Li et al. 2007; Meng et al. 2005). Compared with images presented in those papers, *mopB* mutants have similar morphologies to *fimA* and *pilO* double gene-disrupted strain (DM12 mutant) with no pili. Consistent with our observations, *ompA* mutation in the *E. coli* K1 strain RS218 results in loss of type I fimbriae, which is part of the reason for decreased abilities of OmpA mutant to bind host cells (Teng et al. 2006). Twitching motility is an important factor for the virulence of *X. fastidiosa* (Burdman et al. 2011; Chatterjee et al. 2008), which occurs by the extension, tethering, and then, retraction of type IV pili (Mattick 2002). The colony fringe width and cell twitching motility speed (Fig. 3) demonstrated that *mopB* deletion caused loss of twitching motility. The biogenesis of type IV pili has been extensively studied in several bacteria, such as *P. aeruginosa* and *Myxococcus xanthus*. At least 12 proteins are involved in assembly of a single type IV pilus, and a number of other proteins are related with function and regulation of type IV pili (Burrows 2012; Craig and Li 2008). PilA, the basic structural unit of the type IV pilus filament, is secreted to the cell surface through the outer membrane secretory apparatus. The secretory apparatus consisted of the OMP PilQ and lipoprotein PilF (Burrows 2012; Craig and Li 2008). We propose that deficiency of MopB may affect the outer membrane integrity, then influence the structure or function, or both, of the outer membrane secretory apparatus that causes a defect on pilus biogenesis. MopB is the most abundant protein in the outer membrane of *X. fastidiosa* (Dandekar et al. 2012); therefore, its deficiency may affect membrane integrity. Chen et al. (2010) reported that absence of MopB in *Xanthomonas campestris* pv. *campestris* led to a less-compact outer membrane and a looser connection between inner and outer membranes. Moreover, a previous report (Belaaouaj et al. 2000) indicated that one of the mechanisms of neutrophil elastase-mediated killing of *E. coli* is degradation of OmpA, leading to loss of membrane integrity.

In greenhouse experiments, *Nicotiana tabacum* was used as an experimental host of *X. fastidiosa*, instead of the natural host grapevine, to assess virulence of WT strains and *mopB* mutants. A series of studies established and improved the tobacco system for evaluating virulence of *X. fastidiosa* and investigating *X. fastidiosa*-host interactions (Alves et al. 2003; Francis et al. 2008; Lopes et al. 2000). These studies have shown that *N. tabacum* inoculated with PD-associated strains expressed leaf scorch and leaf cupping or curling symptoms (Oliver et al. 2014). The typical symptom, leaf scorch, in *N. tabacum* was similar to that in PD-affected grapevine (Francis et al. 2008). In addition, *X. fastidiosa* reisolated from symptomatic *N. tabacum* can cause PD symptoms in grapevine, including distinctive leaf scorch and typical 'match stick' (Francis et al. 2008). To evaluate virulence of *X. fastidiosa* PD strains, two aspects were considered: i) percentage of plants that exhibited leaf scorch and ii) percentage of scorched leaves in a symptomatic plant. Thus, strain virulence was established based on the severity and incidence of leaf scorch symptoms. A detailed symptoms evaluation standard for various *X. fastidiosa* strains in *N. tabacum* has been reported in a previously study (Oliver et al. 2014).

Homologs of *mopB* in the plant pathogen *Xanthomonas campestris* pv. *campestris* (Chen et al. 2010) and in the opportunistic pathogen *Stenotrophomonas maltophilia* (Chen et al. 2015) are required for pathogenesis. In our in-plant experiments, *mopB* mutants had reduced virulence in both *X. fastidiosa* strains. Deletion of *mopB* slowed disease development and reduced disease severity in tobacco plants. Using qPCR for bacterial population assessment, *mopB* deletion had no effect on *X. fastidiosa* population in the leaf petiole, showing that the mutants can still colonize the plants. Previous studies from our group indicated no correlation between the *X. fastidiosa* population size in petioles and symptom development in leaves (Oliver et al. 2014). Also, in the study of *X. fastidiosa* movement within grapevines, no significant difference in population size of *X. fastidiosa* between susceptible and resistant cultivars was detected in the petioles (Baccari and Lindow 2011). Given the high concentrations of Ca in xylem sap of tobacco (approximately 2.75 mM, *unpublished*), we speculate that biofilm would be partially restored and, therefore, the delayed symptoms are driven mainly by loss of twitching in these mutants.

Taken together, these results support the critical role of twitching in rapid development of disease symptoms caused by *X. fastidiosa*. In addition, our studies uncovered a role for MopB in both biofilm formation and twitching and therefore added mechanistic detail to the virulence mechanism and further supports use of MopB as a possible target for disease control. Although no clear role of MopB was found here in relation to the Ca response previously described in *X. fastidiosa* (Cruz et al. 2012), the EF-hand motif in the C-terminus of MopB that is predicted to be located in the periplasmic space may interact with Ca to perform other functions, therefore further experiments will be required to uncover the importance of the EF-hand motif in MopB.

MATERIALS AND METHODS

Bacterial strains, plasmids, primers, and culture conditions.

Bacterial strains and plasmids used in this study are listed in Table 1. All primers (Table 2) were designed by PrimerQuest software (Integrated DNA Technologies). *X. fastidiosa* type strain Temecula and a more aggressive strain, WM1-1 (Oliver et al. 2014; Parker et al. 2012), were used as the WT strains. All *X. fastidiosa* strains and mutants were cultured on PW or PD3

plates (Davis et al. 1981) or PD2 broth (Davis et al. 1980) at 28°C. All cell suspensions of *X. fastidiosa* used in this study were prepared as follows. The 7-day-old bacterial cultures were scraped from PW agar medium plates, were suspended in PD2 broth, and were diluted to an optical density at 600 nm (OD₆₀₀) of 1.0. All *E. coli* strains were cultured on Luria-Bertani (LB) (Difco, BD Company) plates or in LB broth. To select transformants, 100 µg of ampicillin and 50 µg of kanamycin per liter were used.

Identification of MopB in silico and construction of mutants.

PD1709 was identified in silico using the BLASTP program from NCBI, using MopB from *Xanthomonas campestris* pv. *campestris* ATCC 33913 (accession number AAM40245) as a query and searching limited in *X. fastidiosa*.

Conserved domains were identified employing the Interpro program from the European Molecular Biology Laboratory-European Bioinformatics Institute. Two-dimensional protein structure was predicted using the web-based PRED-TMBB software (Bagos et al. 2004).

Natural competence and homologous recombination were used to generate *X. fastidiosa* Temecula and WM1-1 *mopB* (PD1709) null mutants. The *mopB* mutant in Temecula was named *mopB*(T) and in WM1-1 was named *mopB*(W).

mopB knockout vectors. Genomic DNA of WT strain Temecula was extracted using a modified cetyltrimethylammonium bromide (CTAB) protocol (Doyle and Doyle

1987). For the construction of the *mopB* knockout vector, 800-bp upstream and downstream fragments flanking the *mopB* open reading frame were amplified by PCR with two primer pairs: *mopB*-U-F/*mopB*-U-R and *mopB*-D-F/*mopB*-D-R. *AscI* restriction site sequences were added to the 5' ends of *mopB*-UP-R and *mopB*-D-F primers. The PCR products were digested with *AscI* and were ligated with T4 ligase (Thermo Fisher Scientific). The ligated product was cloned into pJET1.2/blunt cloning vector (Thermo Fisher Scientific). The resulting plasmid, pJET_ *mopB*, was transformed into *E. coli* NEB 5-α (New England Biolabs), and positive clones were selected on a LB medium plate with ampicillin (100 µg/ml). A kanamycin cassette was amplified from pUC4K with primers containing the *AscI* restriction site sequence (Table 2). pJET_ *mopB* was digested with *AscI* and was dephosphorylated with shrimp alkaline phosphatase (Affymetrix, USB). The kanamycin segment was also digested with *AscI* and was ligated to the digested pJET_ *mopB* by T4 ligase. The final plasmid pJET_ *mopB*_Kan was transformed into *E. coli* NEB 5-α, and positive clones were selected on an LB medium plate with kanamycin (50 µg/ml).

Transformation. *mopB* knockout vector pJET_ *mopB*_Kan was transformed into *X. fastidiosa* Temecula and WM1-1 by taking advantage of the natural competence of the bacteria (Kandel et al. 2017; Kung and Almeida 2011). Briefly, pJET_ *mopB*_Kan was transformed in *E. coli* EAM1 to allow in-vivo methylation of the plasmid (Matsumoto and Igo 2010). A 10-µl cell suspension (OD₆₀₀ = 0.25) of WT strain was mixed with 5 µg of methylated

Table 1. Strains and plasmids used in this study

Strains and plasmids	Characteristics ^a	Source
<i>Xylella fastidiosa</i> Temecula	Wild-type <i>Xylella fastidiosa</i>	Van Sluys et al. 2003
<i>Xylella fastidiosa</i> WM1-1	Wild-type <i>X. fastidiosa</i>	Parker et al. 2012
<i>mopB</i> (T)	<i>mopB</i> mutant in <i>X. fastidiosa</i> Temecula, knockout in PD1709, Km ^R	This study
<i>mopB</i> (W)	<i>mopB</i> mutant in <i>X. fastidiosa</i> WM1-1, knockout in PD1709, Km ^R	This study
<i>Escherichia coli</i> NEB 5-α	<i>fhuA2</i> Δ(<i>argF-lacZ</i>)U169 <i>phoA</i> <i>glmV44</i> Φ80 Δ(<i>lacZ</i>)M15 <i>gyrA96</i> <i>recA1</i> <i>relA1</i> <i>endA1</i> <i>thi-1</i> <i>hsdR17</i> , general cloning host	New England Biolabs
<i>E. coli</i> EAM1	DH5α derivative, Sp ^R St ^R <i>attP</i> _{HK022} ::(P _{LacO} -1-PD1607), expressing the methylase (PD1607) of <i>X. fastidiosa</i>	Matsumoto and Igo 2010
pJET1.2	Blunt cloning vector, Amp ^R	Thermo Fisher Scientific
pJET_ <i>mopB</i>	Regions upstream and downstream of <i>mopB</i> cloned into pJET1.2, Amp ^R	This study
pJET_ <i>mopB</i> _Kan	Kanamycin cassette cloned into pJET_ <i>mopB</i> , Amp ^R , Km ^R	This study

^a Km^R, Amp^R, Sp^R, and St^R indicate resistance to kanamycin, ampicillin, spectinomycin, and streptomycin, respectively.

Table 2. Primers used in this study

Primer	Function or target	Sequence ^a	Source
Mutagenesis			
<i>mopB</i> -U-F	Upstream <i>mopB</i>	CGACCTTCTTTCGTATTCTAG	This study
<i>mopB</i> -U-R	Upstream <i>mopB</i>	ACGGGCGCGCCATGCTTGTCACATCCAGACAG	This study
<i>mopB</i> -D-F	Downstream <i>mopB</i>	ACGGGCGCGCCGTAATGTGCTCCATAGCAAAGC	This study
<i>mopB</i> -D-R	Downstream <i>mopB</i>	ACCATTCCTTGGAAGAGTAGA	This study
KanAscIF	Kanamycin cassette	TTAGGCGCGCCGTCTGCCTCGTGAAG	This study
KanAscIR	Kanamycin cassette	AAAGGCGCGCCCAAGCCACGTTGTGT	This study
Confirmation of mutations			
<i>mopB</i> F	<i>mopB</i> gene deletion	GTCCGTGCTGAAGTAGCTTATC	This study
<i>mopB</i> R	<i>mopB</i> gene deletion	CAGGGTAGCGTCTCAAGATTTC	This study
KanF	Kanamycin cassette replace	TCGGGAAGATGCGTGATCTG	This study
KanR	Kanamycin cassette replace	CGCGATAATGTCCGGGCAATC	This study
B-U-F1	Upstream <i>mopB</i> sequencing	CCTTCTTTCGTATTCTAGACAAC	This study
B-U-R1	Upstream <i>mopB</i> sequencing	AGACAGCAGTCCCACAC	This study
B-D-F1	Downstream <i>mopB</i> sequencing	ACGCTGTAACGCATCTTCTATAC	This study
B-D-R1	Downstream <i>mopB</i> sequencing	ATATTCTCATATGCATTGA	This study
B-UK-F	Kanamycin cassette sequencing	TGTGTGGGACTGCTGTCT	This study
B-DK-R	Kanamycin cassette sequencing	ATCACTGTCCACCTCACATCG	This study
Quantification of <i>Xylella fastidiosa</i>			
HL5	Detection of <i>X. fastidiosa</i>	AAGGCAATAAACGCGCACTA	Francis et al. 2006
HL6	Detection of <i>X. fastidiosa</i>	GGTTTGTCTGACTGGCAACA	Francis et al. 2006
HLP	Detection of <i>X. fastidiosa</i>	FAM-TGGCAGGCAGCAACGATACGGCT-BHQ	Francis et al. 2006

^a *AscI* restriction site is underlined.

pJET_mopB_Kan that was isolated from *E. coli* EAM1 and was spotted on a PD3 medium plate. After cells and DNA settled for an hour, the plates were incubated at 28°C for 3 days. The spot was scraped and was suspended in 250 µl of PD2, and serial dilutions were made. Each dilution (150 µl) was plated on a PW medium plate with kanamycin (50 µg/ml). Plates were incubated at 28°C until transformants were visible (approximately 10 days).

Confirmation of mopB null mutants. For DNA analysis, DNA was extracted from the resulting transformants in Temecula and WM1-1 backgrounds using the CTAB protocol mentioned above. Primer pairs mopBF/mopBR and KanF/KanR, designed from the *mopB* coding region and kanamycin cassette, were used to confirm the deletion of the *mopB* gene. Primer pairs mopB-U-F1/mopB-U-R1, mopB-D-F1/mopB-D-R1, and B-UK-F/B-DK-R were used to sequence the homologous recombination region (Eurofins MWG Operon). Sequences were matched against the *X. fastidiosa* Temecula genome (GenBank), using BLAST to confirm the correct insertion of the kanamycin cassette and the absence of any sequence mismatches.

For SDS-PAGE and protein identification, *X. fastidiosa* strain was washed three times in cold TM buffer (10 mM Tris, pH 8.0, containing 8 mM MgSO₄) and was suspended in 5 ml of fresh cold TM buffer (Chen et al. 2010). The suspension (OD₆₀₀ = 2.0) was sonicated by an ultrasonic cell disruptor. Bacterial proteins were separated by 12% SDS-PAGE, followed by staining of protein bands with Coomassie brilliant blue R-250 (Schagger 2006). The target protein band was excised from the gel and was subjected to liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis (Mass Spectrometry Core in Department of Chemistry and Biochemistry, Auburn University). Bands were identified using X!tandem (Fenyö and Beavis 2003) from the Global Protein Machine website.

Planktonic growth and biofilm formation in media with different Ca concentrations.

Planktonic growth and biofilm formation in 96-well plates was quantified according to methods previously described, with some modifications (Cruz et al. 2012). Briefly, in a 96-well plate, all wells were filled with 190 µl of either i) PD2 broth (PD2), which has a Ca concentration of approximately 0.02 mM (Cobine et al. 2013), ii) PD2 broth supplemented with 4 mM CaCl₂ (4Ca), which Ca concentration is very close to that of the grapevine xylem fluid (Cobine et al. 2013), iii) 50% Sap (*Vitis vinifera* 'Chardonnay' grape xylem fluid mixed with an equal volume of PD2 broth), and iv) full-strength Sap. Grape sap was collected as previously described (Kandel et al. 2016). Four microtiter plates were included in each independent experiment. Ten microliters of cell suspension (OD₆₀₀ = 1.0) were inoculated in the 190 µl of medium contained in each well. For each medium, each strain was inoculated in 12 wells, considered as repetitions. Uninoculated media were used as control. The four microtiter plates were incubated at 28°C and were shaken at 150 rpm for 7 days. The OD₆₀₀ values were recorded daily, which were considered as planktonic growth. On the seventh day, the planktonic phase was transferred to a new microtiter plate. The original plate was rinsed three times with sterile Milli-Q water. Biofilm was stained with 250 µl of 0.1% crystal violet for 20 min and was rinsed with distilled water as described above. Then, 210 µl of 6:4 (vol/vol) acetone/ethanol was added and the plates were agitated at 150 rpm for 5 min. The OD₆₀₀ of the resulting acetone/ethanol solution was measured. Three independent experiments were performed.

Bacterial cell-cell aggregation analysis.

The cell-cell aggregation assay was performed as previously described (Guilhabert and Kirkpatrick 2005). Cell suspension (200 µl; OD₆₀₀ = 1.0) was added into a 50-ml conical tube filled with 10 ml of PD2 broth or PD2 broth supplemented with 4 mM

CaCl₂. Tubes were incubated at 28°C without shaking. Five days after incubation, all tubes were gently vortexed at low speed. After tubes were allowed to stand for 20 min, the OD₆₀₀ of supernatant (OD_S) was measured. The supernatant was returned to the original tube, whole culture was homogenized vigorously by pipetting and OD₆₀₀ (OD_T) was again measured. The relative percentage of aggregated cells was calculated using the formula: Cell aggregation rate = [(OD_T – OD_S) × 100]/OD_T (Burdman et al. 1998). Three replicates for each strain in each medium were used and two independent experiments were performed.

Evaluation of surface attachment in microfluidic chambers.

Adhesion force is a key parameter that reflects the strength of cell attachment to surfaces. This parameter was evaluated using microfluidic chambers, as previously described (Cruz et al. 2012; De La Fuente et al. 2007). The microfluidic chamber consists of a molded polydimethylsiloxane (PDMS) body and a glass microscope slide. There are two parallel microchannels (80 µm wide × 3.7 cm long × 50 µm deep) on one surface of the PDMS body, and this surface was sealed with a glass slide. Two inlets and one outlet for each channel were used to allow media and bacterial suspension to enter the chamber separately and flow out together. The two channels can be simultaneously monitored under microscope.

X. fastidiosa Temecula and *mopB*(T) were suspended in PD2 broth. A 5-ml glass syringe was filled with PD2 broth, while a 1-ml plastic syringe was filled with Temecula cell suspension. Both PD2 broth and Temecula cell suspension were introduced into one microchannel by two automatic syringe pumps (Pico Plus; Harvard Apparatus). Accordingly, PD2 broth and the *mopB*(T) cell suspension were introduced into a parallel and independent microchannel in the same chamber. The flow rate of PD2 broth and bacterial suspensions were also controlled by the syringe pumps. The microfluidic chambers were mounted onto a Nikon Eclipse Ti inverted microscope (Nikon) and were observed under a 40× objective, using Nomarski differential interference contrast optics and phase contrast. After the bacterial cells entered the channels, media flow speed was maintained at 0.25 µl min⁻¹ for approximately 2 h. When enough cells attached to the slide surface, media speed was adjusted to 1.0 µl min⁻¹ for 1 h to remove nonattached cells. Media flow was then sequentially increased every 1 min by 10 µl/min from 1 to 160 µl/min, to gradually remove attached cells. During this process, time-lapse microscopy images were acquired every 5 s, using a Nikon DS-Q1 digital camera (Nikon) controlled by NIS-Elements Advanced Research 3.01 (Nikon). The number of attached cells remaining in each frame was counted. Cell adhesion force was calculated as previously described (De La Fuente et al. 2007). Briefly, the fraction of the cells that detached from the chamber surface at each flow rate as a function of the drag force was plotted. Drag force is the total force exerted on the bacterium by flow (De La Fuente et al. 2007). Adhesion force of Temecula and *mopB*(T) was calculated through integration of each curve that was plotted. Three independent microfluidic chamber experiments were performed.

Quantification of twitching motility.

Twitching motility of *X. fastidiosa* strains was evaluated on PW medium plate with different levels of CaCl₂ supplementation (2 and 4 mM), as previously described (Cruz et al. 2012). For each strain, bacterial cells were spotted in quadruplicate on triplicate plates. After incubating at 28°C for 2 days, the peripheral fringe of each spot was observed under 40× phase-contrast optics on a Nikon Eclipse Ti inverted microscope (Nikon). The width of peripheral fringe was quantified using NIS-Elements Advanced Research 3.01 (Nikon). Three measurements were taken for each spot and three independent experiments were performed.

The twitching speed of Temecula and *mopB*(T) was assessed by microfluidic chamber, as previously described (Cruz et al. 2012; De La Fuente et al. 2007). Cell suspension of Temecula and *mopB*(T) were separately introduced into different channels of the chamber. Media flow rate was controlled at 0.25 $\mu\text{L min}^{-1}$ for 2 h to allow cells attached on the surface. Time-lapse images were then acquired every 30 s for 2 or 3 h with a Nikon DS-Q1 digital camera. Using NIS-Elements Advanced Research 3.01, the upstream movement of bacterial cells was quantified according to their position frame by frame. Three independent experiments were performed.

Transmission electron microscopy.

X. fastidiosa strains were spread on PW medium plate for 2 days. Colonies were scraped and suspended in 200 μL of distilled water. A 10- μL cell suspension was deposited on Formvar-coated grids and was subsequently stained with 50 μL of phosphotungstic acid. After 2 min, the excess liquid on the grids was removed, and the grids were air dried for 1 min and were observed on a Zeiss EM 10 transmission electron microscope (Carl Zeiss). Two independent experiments were performed.

Greenhouse experiments.

All *X. fastidiosa* strains were inoculated into tobacco plants (*Nicotiana tabacum* 'Petite Havana SR1', plant introduction number 552516) to evaluate their virulence, as previously described (De La Fuente et al. 2013). Seeds were provided by the United States Department of Agriculture Germplasm Resources Information Network. After seeds were germinated in SunshineH Mix #8 (Sun Gro Horticulture Canada Ltd.), seedlings were transplanted in 4.5-in square pots and were grown in the greenhouse at 20 to 25°C with natural sunlight. Plants were watered regularly. Fertilizer (Peter's Professional 20-10-20 Peat-Lite Special; The Scotts Company) was applied one time at 40 to 45 days posttransplant. When tobacco plants had five to six true leaves, the top of the stem and the leaves were removed. The lowest three of the true leaves were kept and were inoculated with *X. fastidiosa* cells suspended in 1.5 ml of succinate-citrate phosphate buffer (OD₆₀₀ of 1.0) by a 1-ml tuberculin syringe. Eight plants were inoculated for each *X. fastidiosa* strain, and eight control plants were only inoculated with buffer. Two independent experiments were performed.

Following inoculation, plants were grown in the greenhouse for two or three months, until leaf scorch symptom appeared. Plants symptoms were then assessed weekly for 4 weeks. Symptoms on leaves above the site where the stem was cut were recorded. Disease incidence was represented by the percentage of infected plants in the eight plants inoculated with the same strain.

To determine if inoculated plants became infected with *X. fastidiosa*, a qPCR analysis was performed as described previously (De La Fuente et al. 2013; Oliver et al. 2014). Briefly, leaves were collected at the end of the fourth week of symptom monitoring. For each strain, three infected plants were used for leaf collection. Four leaves above the inoculation point were collected from each plant and sections of the lower petiole were excised. Total DNA of the petioles was extracted using a modified CTAB protocol (Doyle and Doyle 1987) and *X. fastidiosa* CFU per gram of extracted petiole tissue was quantified, using qPCR and a standard curve as previously described (De La Fuente et al. 2013).

In addition, *X. fastidiosa* was isolated from infected plants, using a method described previously (Parker et al. 2012). Briefly, a short segment of infected plant stem was cut to tiny pieces after surface sterilization. The stem was soaked in PD2 broth at 28°C with shaking at 150 rpm for 1 h. The supernatant was diluted and was spread-plated on agar PW medium and PW with kanamycin (50 $\mu\text{g ml}^{-1}$).

Statistical analysis.

Data from biofilm production in 96-well plates, fringe width, and cell aggregation were analyzed by SAS 9.4 (SAS Institute Inc.) using GLIMMIX procedure, and means were separated by the Tukey-Kramer method ($P < 0.05$). To compare the difference of adhesion force, twitching motility speed, and the area under the disease progress curve between mutant and corresponding WT strain, data were analyzed by *t* test ($P < 0.05$).

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AUTHOR-RECOMMENDED INTERNET RESOURCES

European Molecular Biology Laboratory-European Bioinformatics Institute Interpro program: <https://www.ebi.ac.uk/interpro>
 NCBI BLASTP program: <https://blast.ncbi.nlm.nih.gov/Blast.cgi>
 PRED-TMBB server: <http://bioinformatics.biol.uoa.gr/PRED-TMBB>