Effect of calcium on biofilm development and role of *mopB* and *msrA* in virulence of *Xylella fastidiosa*

by

Hongyu Chen

A thesis submitted to the Graduate Faculty of
Auburn University
in partial fulfillment of the
requirements for the Degree of
Master of Science

Auburn, Alabama
August 6, 2016

Key words: *Xylella fastidiosa*, Calcium, Biofilm, Virulence, *mopB*, *msrA*

Approved by

Leonardo De La Fuente, Chair, Associate Professor of Plant Pathology
Jeffrey J. Coleman, Assistant Professor of Plant Pathology
Covadonga R. Arias, Professor of Aquaculture and Aquatic Sciences

Abstract

Xylella fastidiosa is the causal agent of many diseases on economically important plants. Pierce's disease of grape (PD) and citrus variegated chlorosis (CVC) are among the most important diseases caused by *X. fastidiosa*. Disease symptoms including marginal leaf scorch and shriveling of fruit are associated with the blockage of water flow in xylem vessels, caused by extensive colonization of the host xylem system by *X. fastidiosa*. Twitching motility and biofilm formation are considered virulence traits of this pathogen.

Research conducted in our laboratory has proven that calcium (Ca), a mineral nutrient in xylem sap, influences virulence traits of *X. fastidiosa*. Ca increases biofilm formation by affecting the initial stages of biofilm development. However, the effect of Ca on the later stages of biofilm development have not been characterized. In this present study, biofilm and planktonic growth over time of *X. fastidiosa* 'Temecula' cultured in different Ca-amended media was quantified. Results show that when cells are grown in non-Ca amended medium (0.02mM Ca), by 96 hours they have already reached the dispersion stage, while cells in Ca-supplemented medium (4 mM) are still forming dense, mature biofilms over the same time period. These results indicate that Ca supplementation contributes to prolong biofilm development of *X. fastidiosa* strain 'Temecula'.

In addition, in order to provide information for further understanding the mechanism of *X. fastidiosa* response to Ca, two putative Ca-related genes *mopB* and *msrA* were selected to focus

our studies. The role of these two genes in virulence traits in vitro and in disease development in vivo was evaluated. *mopB* and *msrA* mutants in two *X. fastidiosa* strains 'Temecula' and 'WM1-1' were constructed by site-directed mutagenesis. All knockouts had similar response to Ca supplementation as the respective WT strains, indicating that these particular genes are not responsible for the increase in biofilm and/or movement observed in WT strains after Ca supplementation. Nevertheless, *mopB* mutants in both background strains were impaired in surface attachment, biofilm formation, and twitching motility. In addition, *mopB* mutants were impaired in pilus formation as observed by electron microscopy. *mopB* mutants in both backgrounds showed reduced virulence when tested on tobacco as a host under greenhouse conditions. In contrast, *msrA* mutation in either background strain had no effect on virulence traits and disease development. These results suggest that outer membrane protein MopB is required for biofilm formation, motility, pilus biogenesis and virulence of *X. fastidiosa*, but *msrA* gene play no role in these processes under the conditions evaluated.

Overall, these results show that Ca can prolong biofilm development of *X. fastidiosa*, which is important to understand the disease progression in plants. MopB is important for virulence of *X. fastidiosa* but MsrA is not a major virulence determinant for this pathogen. Both genes are not responsible for Ca-enhanced biofilm formation and/or twitching motility in *X. fastidiosa*.

Acknowledgments

I would like to extend my gratitude to everyone that helped me in this journey. I want to express my deepest appreciation to my advisor, Dr. Leonardo De La Fuente, for giving me the opportunity to join his research group and for his guidance and encouragement throughout this project. Without his patience and endless support, my thesis would not have been possible. Thanks to Dr. Covadonga R. Arias and Dr. Jeffrey J. Coleman for serving on my graduate committee offering their suggestions and helps.

Special thanks to Jennifer Parker for help me to become familiar with all aspects in the lab; to Luisa Cruz and Prem Kandel for construction of *mopB* and *msrA* mutants, which are important materials in my thesis project. Thanks to all my labmates: Fernando Navarrete, Jonathan Oliver, Sy Mamadou Traore, Balapuwaduge Mendis, Eber Feliciano for their useful suggestions and discussions about the experiments.

I would also like to extend a special thanks to my wife, Ni Xiang, and my family for their encouragement and unconditional support.

Table of Contents

List of Tables

List of Illustrations

Chapter 1: Literature review

Xylella fastidiosa

Xylella fastidiosa have been reported as the causal agent of many diseases on plants including grapevine, citrus, olive, alfalfa, peach, plum, almond, elm, coffee, sycamore, oak, maple and pear [1, 2]. Pierce's disease of grape (PD) and citrus variegated chlorosis (CVC) are among the most important diseases caused by *X. fastidiosa* [2]. PD is a constant threat to the wine industry of the United States, causing millions of dollars in losses every year in California and other states in southern areas of the country [3]. CVC was present in approximately 40% of citrus plants in Brazil and causes losses of around 120 million US dollars to the Brazilian citrus industry annually [4]. Both diseases have received more attention than other diseases caused by this pathogen.

Xylella fastidiosa was first discovered associated with PD in 1973 and was first grown in axenic culture in 1978 [5]. This pathogen is a fastidious, gram-negative, and strictly aerobic bacterium with a rod shape. The optimum growth temperature for this slow growing bacterium is 26-28 ℃ [6]. This bacterium is non-flagellated but has two types of pili, named type I and type IV, which are anchored on one pole of the bacterial cell. The type I pilus is associated with bacterial adhesion and the type IV pilus contributes to twitching motility [7]. In its natural environment, *X. fastidiosa* only colonizes two habitats, the xylem vessels of plants and the foregut of xylem-sap feeding insects [2].

Taxonomy and Geographical Distribution

Xylella fastidiosa is a gamma-proteobacterium in the order Xanthomonadales, family Xanthomonadaceae, and genus *Xylella* [8]. Based on DNA hybridization and multi-locus sequence typing data, this species has been divided into four subspecies: *X. fastidiosa* subsp. *fastidiosa*, *X. fastidiosa* subsp. *multiplex*, *X. fastidiosa* subsp. *sandyi*, and *X. fastidiosa* subsp. *pauca* [9, 10]. Different subspecies infect different hosts and have a various geographical distribution. *X. fastidiosa* subsp. *fastidiosa* is presumed to be native to Central America, and this subspecies infects grape, alfalfa, almond and maple; *X. fastidiosa* subsp. *multiplex* is native to temperate and subtropical North America, and this subspecies infects peach, plum, sycamore, elm, blueberry and almond; *X. fastidiosa* subsp. *sandyi* has only been detected in southern regions of the United States, and this subspecies infects oleander; *X. fastidiosa* subsp. *pauca* is native to South America, and this subspecies infects citrus and coffee [9-11]. However, geographical distribution and host range of these subspecies never stop changing. Recently, pure cultures of *X. fastidiosa* were isolated from naturally infected olive and oleander plants in Apulia, Italy; and these isolates belong to the subspecies *pauca* [12]. Many factors contribute to *X. fastidiosa* being introduced into other areas and countries. Natural competence and recombination in this pathogen are associated with adaption to new plant hosts.

Colonization of host plants

Xylella fastidiosa is different in the infection process compared to other plant vascular pathogenic bacteria such as *Xanthomonas campestris*, *Ralstonia solanacearum* or *Erwinia amylovora*. These bacteria initially infect one of the plant organs, such as leaf, root or flower,

and then these bacteria eventually move into the vascular systems [13]. *X. fastidiosa* needs sap-feeding insects, such as sharpshooters, leafhoppers, and spittlebugs, for inoculation into host plants. The bacteria are directly injected into xylem vessels during the process of sap feeding. This bacterium appears to be exclusively xylem-limited; it cannot spread in other tissues through xylem vessels.

From inoculation at the feeding site to systematic colonization of xylem vessels, *X. fastidiosa* needs to overcome two obstacles: the water flow inside the xylem and pit membranes (PM) between neighbor xylem vessels. The bacteria being translocated upward in the host xylem network carried by flow, is easy to understand; however, how the bacteria move against the direction of the water stream in the vessel was a puzzling question. Meng et al. [14] using a microfluidic chamber to mimic the plant xylem vessel, studied upstream migration of *X. fastidiosa*, which indicated that twitching motility mediated by type IV pili contributes to the movement of the bacteria against flow direction. Twitching occurs by the extension, tethering and then retraction of polar type IV pili [15].

PM is the connection between neighboring vessel elements, and is a primary cell wall not covered with the secondary wall; which allows water to move from one vessel to the next, but limits the spread of vascular pathogens and air embolisms [16]. The movement of *X. fastidiosa* from one xylem vessel to another not only requires twitching motility but also depends on its ability to disrupt PM. Perez-Donoso et al. [17] using cell wall-degrading enzymes produced by *X. fastidiosa*, treated the PM of grapevine, which enlarged the pore size of the PM. This study indicated a potential ability of *X. fastidiosa* to dissolve the PM.

When *X. fastidiosa* colonizes throughout the xylem system, the distribution of the bacterial cells is not homogenous in the plant xylem. In grapevine, more *X. fastidiosa* cells accumulated in symptomatic leaf veins and petioles than in the stem [18].

Development of disease symptom

The exact pathogenicity mechanisms of *X. fastidiosa* are yet to be fully understood. However, several studies suggest that disease is associated with extensive colonization of this pathogen in the host plant xylem. Symptoms including marginal leaf scorch and shriveling of fruit are consequence of physiological responses to water deficit caused by vessel blockage [11]. Once the pathogen is inoculated into the host plant by the insect vector, cells attach to the surface of xylem vessels; with multiplication of cells and production of various extracellular compounds, biofilms are formed that lead to blockage of vessels [2, 19]. In addition, structural modifications of the host plant in response to pathogen infection also caused the occlusion of the xylem vessels. Sun et al. [20] investigated the xylem structure of healthy and *X. fastidiosa*-infected grapevine, and they found that infected grapevine form three types of materials (tyloses, pectin-rich gels, and crystals), and each type appeared to be able to occlude vessels. Overall, xylem vessels of host plants were blocked by bacterial cells and/or materials of plant origin, which causes deficiencies of water and nutrition transportation.

Virulence of *Xylella fastidiosa*

Disease caused by *X. fastidiosa* appears to be a consequence of bacterial multiplication and movement within the host plant xylem system [2, 21]. Cell attachment, migration within the plant, and biofilm formation are considered determinants for plant infection. Fimbrial and afimbrial adhesins are important for these processes. Type I and type IV pili are the most important fimbrial adhesins produced by *X. fastidiosa*. The length of type I pili are 0.4 - 1.0 μm; type IV pili are 1.0 - 5.8 μm [14]. Previous studies reported that the *X. fastidiosa fimA* null mutant (without type I pili) significantly reduce the strength of surface attachment [22]. Many genes have been reported to be essential for biogenesis of type IV pili. The genome of *X. fastidiosa* contains more than 25 putative *pil* genes that are associated with type IV pilus formation and twitching motility [7]. For example, *pilA* encode the major structural protein of the type IV pilus; *pilE, pilV, pilW,* and *pilY1* encode minor proteins involved in the formation of the base and tip of the pilus; *pilB, pilC, pilD, pilF, pilM, pilN, pilO,pilP*, and *pilQ* are required for the pilus assembly and retraction [7]. Afimbrial adhesins are surface proteins that are located in the outer membrane; hemagglutinins are typical proteins in this group of adhesins. HxfA and HxfB are two hemagglutinins of *X. fastidiosa,* which are present in the outer membrane and vesicles [23]. Deletion mutations of the *hxfA* or *hxfB* gene, result in a loss of cell to cell aggregation and biofilm formation [24].

Biofilms are bacterial assemblages with a complex architecture, composed by bacterial cells and self-produced extracellular substances, such as exopolysaccharides (EPS), proteins, lipids, and extracellular DNA [25]. Biofilm formation is a well-known behavior in bacteria, and this

behavior of *X. fastidiosa* is critical for development of symptoms. Biofilm formation of *X. fastidiosa* can be divided into five stages, including reversible adhesion, aggregation and production of EPS resulting in irreversible attachment, early maturation, mature biofilm, and dispersion [26, 27]. The structural integrity of biofilms is associated with EPS, which also contributes to the virulence of *X. fastidiosa*. Killiny et al. [28] assessed mutations of two genes involved in synthesis of EPS in *X. fastidiosa,* which demonstrated that EPS is essential for pathogenicity and insect transmission.

Similar to other bacteria, quorum sensing is important for pathogenicity of *X. fastidiosa*. Diffusible signaling factor (DSF) is a small fatty acid molecule that mediates cell-to-cell signaling in bacteria. DSF is encoded by the *rpf* gene cluster, which regulates bacterial virulence [2]. Cyclic di-GMP is an intracellular signal messenger that is modulated by DSF to regulate biofilm formation, plant virulence, and insect transmission [29]. In addition, several proteins involved in secretion systems (TolC, LesA), response regulators (XhpT), autotransporter (XatA), and membrane transportation (TonB) have been reported to be related with virulence of *X. fastidiosa* [30-34].

Minerals in xylem sap

The main function of the xylem is transportation of water and mineral nutrition from roots to leaves. Metal elements, such as copper (Cu), iron (Fe), zinc (Zn), calcium (Ca), magnesium (Mg) and others, are important components of xylem sap, which involve in many critical biological processes including oxygen transport, cell growth and differentiation, and protection against oxidative stress [35]. Metal elements generally serve structural and/or catalytic functions [36];

therefore, they are necessary for the survival of all living organisms. Although these elements are required, they are under tight homeostatic control because they have the potential to be toxic at high concentrations. Plants have the ability to regulate the concentration and distribution of all metals at a cellular level. This is important for providing sufficient metals for the development of tissues [37]. Bacteria that infect plant xylem face a challenge for survival that is that they need to recruit the same essential elements which were guarded by the host carefully. Therefore, pathogens must develop strategies to compete with the host for certain elements.

Effect of metal elements on *X. fastidiosa*

Xylella fastidiosa in a host plant is restricted to the xylem vessels. Andersen et al. studied the influence of grapevine xylem fluid chemistry on cells growth and aggregation of *X. fastidiosa*. The results indicated that metal elements including copper (Cu), zinc (Zn), calcium (Ca), and magnesium (Mg) were correlated with planktonic growth and cell aggregation [38].

Copper and Zn appear to be stress factors in xylem sap for *X. fastidiosa* survival. The toxic properties of Cu have been used to attack invading pathogens [39]. Cu resistance of *X. fastidiosa* was evaluated, and the results shown biofilm cells were more resistant to Cu compared to planktonic cells [40]. That study also indicated that the Cu resistance seems to be directly related with the amount of EPS produced by *X. fastidiosa* cells, since EPS can absorb metal cations [40]. In vitro, Cu promotes attachment and aggregation of *X. fastidiosa* at low levels, but it prevents growth at higher concentrations. Zinc reduces the planktonic and biofilm growth, but increases EPS production and adhesion strength of biofilm. Copper and Zn were accumulated at higher

levels in biofilm cells than planktonic ones [37, 41]. In plants, that detoxification of Zn is essential for full virulence of *X. fastidiosa* has been proved, which suggests that host plants through Zn levels limit the growth of this pathogen [42].

Iron is a required mineral nutrient for all organisms and plays an important role in host-bacteria interactions. All microorganism have numerous strategies for Fe acquisition; one of these strategies is the siderophores system [43]. Siderophores are low-molecular-mass Fe chelators that are secreted by bacteria [43]. A former study investigated growth and production of siderophores by *X. fastidiosa* in different Fe concentration media, which demonstrated that *X. fastidiosa* grew less and produced more siderophores in iron-limited conditions [44]. Using DNA microarrays, Zaini et al. [45] analyzed the transcripts of *X. fastidiosa* under different Fe concentrations. The results indicated this metal regulates genes required for type I and type IV pili, and genes involved in the production of colicin V-like bacteriocins. These suggest that Fe affects the survival of *X. fastidiosa* in plants and the pathogenicity determinants of this pathogen.

Calcium is important for *X. fastidiosa*

i. Calcium modulates bacterial phenotypes

Calcium, a critical nutrient element, plays important roles and is required for all organisms. In bacteria, Ca working as a regulator modulates a variety of bacterial phenotypes including motility, extracellular compounds biosynthesis, biofilm formation and others [46, 47].

Calcium involves in the function of flagellum or pilus to affect bacterial motility. Lefèvre et al. [48] reported that Ca mediates the assembly of flagellar sheath of a marine magnetotactic

bacterium MO-1, which is essential for smooth swimming of the cells. In another marine bacterium, *Vibrio parahaemolyticus*, Ca regulates bacterial swarming by repressing the transcription factor CalR, that modulates flagellar gene expression [49]. In addition, Ca enhanced twitching motility of the bacteria *Kingella kingae* and *Pseudomonas aeruginosa* through interaction with a structural protein of type IV pili [50, 51].

Calcium has been shown to have positive and negative effects on biofilm formation of bacteria. Ca affects the biofilm formation of *Pseudoalteromonas* sp. [52] and *P. aeruginosa* [53] through increasing the amount of surface-associated biomass and extracellular matrix material. Recently, Mangwani et al. [54] also reported that Ca increases the extracellular polymeric substances production of *P. mendocina* NR802 and Ca-enhanced biofilm growth of this bacterium in a dose dependent manner. On the contrary, Ca negatively affects biofilm formation of *Vibrio cholerae* by decreasing the expression of genes required for biofilm matrix production [55]. A similar phenotype, Ca-inhibition of biofilm, was also observed in *Staphylococcus aureus*; Ca significantly influenced the architecture and topography of the biofilm [56]. A biofilm-associated protein (Bap) of *S. aureus* possessing a potential Ca-binding motif is responsible for this phenotype; Ca-binding causes a conformational change that renders the protein unable to accomplish its function in cell aggregation and biofilm formation [57].

ii. Calcium influence on virulence traits of *X. fastidiosa*

Commonly, pathogen infection causes physiological changes of host organisms leading to symptom appearance. When host plants were infected with *X. fastidiosa*, the changes of leaf ionome were measured as the plant transitioned from healthy to disease status. The results show

that the pathogen infection causes a significant increase in Ca concentrations prior to the appearance of symptoms [58].

In vitro, Ca was found to accumulate at a higher concentration in biofilm than that in planktonic cells [37]. Leite et al. [59] studied biofilm of *X. fastidiosa in vivo*; and according to genomics information and x-ray microanalyses, they proposed that Ca and Mg work as divalent cation bridges connecting negatively charged thiol groups on bacterial surface and negatively charged xylem vessels. This proposal suggests one model of Ca mediating adhesion and aggregation of *X. fastidiosa*.

Studies of our group have shown that Ca regulates key virulence traits of *X. fastidiosa*. Calcium supplementation was found to increase surface attachment, biofilm formation, and twitching motility of *X. fastidiosa* [60]. The Ca-enhanced twitching motility is associated with the interaction between Ca and a Ca-binding motif in the type IV pilus structural protein, PilY1 [61]. Using tetracycline to treat *X. fastidiosa,* which disrupts *de novo* protein synthesis of the bacterium, Ca supplementation failed to induce an increase in surface attachment of the treated bacterium [60]. This suggests the presence of a regulatory effect of Ca on *X. fastidiosa* in addition to a structural effect. Recently, comparative transcriptome analyses of *X. fastidiosa* biofilm cells grown in batch culture in high level and low level Ca concentration media were conducted by our research group, to identify genes differentially expressed at higher concentrations of Ca. 240 genes associated with adhesion, biofilm formation, motility, peptidoglycan synthesis, regulators, metal homeostasis and others were identified [47]. That study confirmed a regulatory role for Ca in *X. fastidiosa*.

Mechanism of Ca regulatory role in bacteria

Calcium is an ubiquitous intracellular messenger in eukaryotic cells and regulates many important cellular processes including cell cycle, cell differentiation, motility, transport, stress signals and metabolism [62]. This messenger transmits signals from the cell surface to the interior of the cell by transient changes in the intracellular free-Ca concentration as well as the specific speed, magnitude, frequency and spatio-temporal patterns of the changes [46, 63]. Calcium binding proteins, the sensors of Ca, convey the information presented in the changes via phosphorylation events, protein-protein interactions and regulation of gene expression [64].

Compared to the extensive studies of Ca in eukaryotes, the research of the role of Ca in prokaryotes still lags behind. However, during recent years, much evidence indicated that Ca plays a regulatory role in prokaryotes [46]. Former studies reported that Ca affects a number of bacterial processes including maintenance of cell structure, sporulation, heterocyst differentiation, chemotaxis, motility and virulence [46, 62].

i. Calcium homeostasis in bacterial cell

Similar to eukaryotic cells, the intracellular free Ca concentration in bacterial cells is tightly regulated, which was reported ranging from 100 to 300 nM, similar to the levels found in eukaryotic cells [65, 66]. The presence of abilities to control Ca homeostasis are the prerequisite for the regulatory role of Ca. Bacteria possess a number of mechanisms for uptake and extrusion of Ca, such as Ca channels, Ca-translocating ATPases, and electrochemical potential driven Ca transporters [46].

Studies on a molecular level suggest that Ca channels are present in bacteria. A mechanosensitive ion channel MscL was identified in *Synechocystis* sp. PCC 6803. MscL might work as an outward Ca channel that releases Ca from the cell into medium in response to temperature stress conditions [67]. BsYetJ in *Bacillus subtilis* is a Ca leakage channel that can pass extracellular Ca into the cytoplasm, which is homologous to the human hBI-1 channel mediating Ca release from the ER into the cytoplasm [68]. Calcium ATPases are mostly high-affinity Ca pumps that transport this ion from the intracellular to the extracellular environment, and this process consumes the energy stored in ATP [46]. Previous studies have shown that there are two types of Ca ATPases in bacteria, P-type and F-type; where P-type ATPases hydrolyze ATP and F-type ATPases are synthases of ATP [46, 69]. Electrochemical potential driven Ca transporters are mostly low-affinity Ca transport systems using the energy stored in the electrochemical gradient of ions, which can uptake and export the cation [46].

Some proteins that can bind Ca are important for maintenance of intracellular Ca homeostasis, such as the putative EF-hand protein EfhP in *P. aeruginosa* and proteins were identified in *B. subtilis*, which can bind Ca but lacks known Ca binding domains [70, 71]. In several bacteria, membrane bound structures, outer membrane, and periplasm appear to be crucial in the regulation of intracellular Ca concentration. These structures sequester a high concentration Ca and serve as a barrier that prevent Ca from entering the cytoplasm [72, 73].

ii. Calcium binding proteins in bacteria

Recently, a number of Ca binding proteins have been identified in prokaryotic organisms. Most of these proteins contain sequences closely resembling the typical eukaryotic Ca binding motif, such as EF-hand and EF-hand-like motifs, Ca-binding β-roll motif and Ca-binding Greek key motifs [46]. The superfamily of EF-hand proteins is the largest and best characterized group of Ca binding proteins [46]. Four conserved sequence patterns representing EF-hand motifs in bacteria have been reported: DxDxDG [74], Dx[DN]xDGxxD [50], DxDxNxxxD [75], and Dx[DN]xDxxxxxx[DE] [51]. Ca can regulate the surface adhesion and motility of the bacteria *X. fastidiosa*, *Kingella kingae* and *Pseudomonas aeruginosa* through proteins containing EF-hand motifs [50, 51, 61]. According to these sequence patterns, a large number of putative Ca binding proteins have been identified. By searching *X. fastidiosa* 'Temecula' genome, 42 genes were identified that contain one of the four Ca-biding motifs sequence patterns [47]. Seven of these genes were differently expressed at different level of Ca concentration in the media, according to whole transcriptome analysis [47]. However, experimental studies confirming that these proteins bind to Ca are lacking. An exception is the study on the type IV pilus protein PilY1 of *X. fastidiosa* [61], whose Ca binding ability was inferred by homology and indirect evidence from biochemical studies.

iii. Calcium signaling in bacteria

Several lines of indirect evidence indicate the presence of Ca signaling in bacteria. Firstly, Ca-binding proteins were shown to mediate Ca effects in bacteria. CabC is an EF-hand Ca binding protein in *Streptomyces coelicolor*. This protein acts as a Ca ion buffer and is involved in Ca ion mediated regulation of spore germination and aerial hypha formation by controlling the

intracellular Ca concentration [76]. EfhP with an EF-hand domain is a putative Ca binding protein, which is important for Ca homeostasis of *Pseudomonas aeruginosa*. Mutational analysis of EfhP indicated that this protein is required for several Ca mediated processes, including pyocyanin production, oxidative stress resistance and virulence. Similarly, PilY1 is a structural protein of type IV pilus with an EF-hand domain. This protein has responded to Ca-enhanced twitching motility of *P. aeruginosa* and *X. fastidiosa* [50, 61]. A cyanobacterial Ca binding protein CcbP is an acidic amino acid rich protein with two distinct Ca binding domains. This protein mediates Ca regulated heterocyst differentiation of *Anabaena* sp. PCC7120 via changing of intracellular free Ca ion concentration [77].

Additionally, two component systems and regulatory proteins, whose role is to adjust bacterial physiology to environmental stimulations, were shown to respond to Ca. PhoQ kinases are part of the PhoPQ two component system in *Salmonella typhimurium*; this system transcriptionally regulates hundreds of genes encoding the majority of virulence properties. The activity of PhoQ is repressed by divalent cations, such as a Ca ion [78]. A two component system CarRS was identified in *Vibrio cholerae*; this system is controlled by Ca and negatively regulated the biofilm formation of this bacterium [55]. In *Streptococcus pneumoniae,* HrcA is a regulatory protein that represses the transcription of protein folding related operons by binding CIRCE (controlling inverted repeat of chaperone expression) operator sequence. Calcium induces conformational changes affected interaction of HrcA and CIRCE, which influences the thermoresistance of *S. pneumoniae* [79].

Lastly, Ca modulates gene expression in bacteria. Proteome analysis of *Bacillus subtilis* and *Pseudomonas aeruginosa* revealed that the expression of hundreds of genes are modulated by Ca supplementation [71, 80]. Transcriptome analysis showed that Ca can regulate the expression of genes involved in adhesion, biofilm formation and twitching motility of bacteria *V. cholera* and *X. fastidiosa* [47, 55].

Outer membrane protein MopB and methionine sulfoxide reductase MsrA

MopB, a major outer membrane protein, has been reported in *Methylococcus capsulatus*, *Xanthomonas campestris* pv. *campestris* and *X. fastidiosa* [81-83]. Based on sequence analysis, the C-terminal part of MopB is a homologue in the OmpA family; a EF-hand motif, Dx[DN]xDxxxxxx[DE], is also located in this part of the protein [47, 81, 82]. OmpA proteins have been detected in representatives of a wide range of bacteria. In *E.* coli, OmpA plays a structural role in the integrity of the bacterial cell surface [84]. Thus it seems reasonable to assume that MopB may also have a structural role. MopB possessing an EF-hand motif indicated it is a putative Ca-binding protein. This protein appears to be associated with the Ca regulatory role in bacteria. Chen et al. [82] reported that MopB is important for maintaining outer membrane integrity of *Xanthomonas campestris* pv. *campestris*, a phytopathogen that causes black rot in crucifers, and is required for pathogenicity of this pathogen. However, the role of MopB in virulence of *X. fastidiosa* has not been studied. Dandekar et al. [83] identified MopB as a recognition target in an engineered antimicrobial therapeutics approach for protection against *X. fastidiosa*. Nevertheless, the function of this protein remains poorly understood; and it has not been studied whether this protein is involved in a regulatory role of Ca.

Methionine sulfoxide reductase MsrA is encoded by *msrA*, which is an enzyme responsible for repairing oxidative damage to proteins [85]. The substrates of this enzyme are free methionine-sulfoxide and methionine-sulfoxide residues in proteins [86]. MsrA has been reported to be required for full virulence in some pathogenic bacteria; since the enzyme can protect the pathogen against the host defense reaction, in particular against the production of reactive oxygen species [85, 87, 88]. In addition, MsrA has been found to be involved in cell adhesion by pathogenic bacteria [89]. *msrA* mutation in *Streptococcus pneumoniae*, *Neisseria gonorrhoeae*, *Escherichia coli* and *Erwinia chrysanthemi* strains were defective in attachment to the host cell [85, 89]. In *X. fastidiosa*, *msrA* gene expression of biofilm cells are downregulated approximately 3-fold in Ca supplemented media compare to that in non-supplemented media [47]. Additionally, based on genomics and x-ray microanalysis, it has been proposed that MsrA helps to maintain thiol groups on the bacterial surface, and these thiol groups interact with Ca and Mg (magnesium ion) to form divalent cation bridges mediating the bacterial aggregation and adhesion [59]. Accordingly, MsrA seems to involve in Ca-regulated virulence traits of *X. fastidiosa*, however, the putative function of MsrA in *X. fastidiosa* has not been experimentally confirmed.

Summary

Xylella fastidiosa, a pathogenic bacterium, causes diseases on many economically important crops. *X. fastidiosa* is restricted to the plant xylem vessels, and the pathogen obtains almost all the required nutrients from the xylem sap; thus components of xylem sap are critical for growth

and virulence of this pathogen. Ca, as an important mineral nutrient in xylem sap, affects virulence traits of *X. fastidiosa* including surface attachment, biofilm formation and twitching motility. Understanding the mechanisms of these effects can help us find practical methods to control the diseases caused by *X. fastidiosa*.

Previous studies indicate a regulatory role of Ca in prokaryotes. There are two main mechanisms by which Ca can influence protein activity and therefore bacterial metabolism: Ca modulates gene expression and Ca directly interacts with Ca-binding protein to affect the physiology of bacteria. MopB possess a putative Ca-binding motif, *msrA* is regulated by Ca, and both genes seem to relate to virulence of *X. fastidiosa*. These two proteins were chosen as the focus of this study that was based on the following hypothesis and objective:

Hypothesis

Virulence traits of *X. fastidiosa* affected by Ca, an important mineral nutrient in xylem sap, are associated with the function of EF-hand protein MopB and Ca- regulated gene *msrA*.

Overall Objective

Evaluate the continued effect of Ca on biofilm and planktonic growth, and elucidate functions of the MopB and MsrA proteins to further understand the mechanism(s) by which Ca regulates virulence traits of *X. fastidiosa*.

Specific Objectives

1, Evaluate the effect of Ca on *X. fastidiosa* biofilm and planktonic growth over time

2, Mutational analysis and characterization of genes encoding an EF-hand protein (MopB) and a Ca-regulated gene (*msrA*) in two *X. fastidiosa* strains.

References

1. Cariddi, C., et al., *Isolation of a Xylella fastidiosa strain infecting olive and oleander in Apulia, Italy.* Journal of Plant Pathology, 2014. **96**(3): p. 1-5.
2. Chatterjee, S., R.P. Almeida, and S. Lindow, *Living in two worlds: the plant and insect lifestyles of Xylella fastidiosa.* Phytopathology, 2008. **46**(1): p. 243.
3. Tumber, K.P., J.M. Alston, and K. Fuller, *Pierce's disease costs California $104 million per year.* California Agriculture, 2014. **68**(1-2).
4. Gonçalves, F., et al., *Relationship between sweet orange yield and intensity of Citrus Variegated Chlorosis.* Plant Pathology, 2012. **61**(4): p. 641-647.
5. Hopkins, D., *Xylella fastidiosa: xylem-limited bacterial pathogen of plants.* Annual review of phytopathology, 1989. **27**(1): p. 271-290.
6. Janse, J. and A. Obradovic, *Xylella fastidiosa: its biology, diagnosis, control and risks.* Journal of Plant Pathology, 2010: p. S35-S48.
7. Li, Y., et al., *Type I and type IV pili of Xylella fastidiosa affect twitching motility, biofilm formation and cell–cell aggregation.* Microbiology, 2007. **153**(3): p. 719-726.
8. Wells, J.M., et al., *Xylella fastidiosa gen. nov., sp. nov: gram-negative, xylem-limited, fastidious plant bacteria related to Xanthomonas spp.* International Journal of Systematic and Evolutionary Microbiology, 1987. **37**(2): p. 136-143.
9. Scally, M., et al., *Multilocus sequence type system for the plant pathogen Xylella fastidiosa and relative contributions of recombination and point mutation to clonal diversity.* Applied and Environmental Microbiology, 2005. **71**(12): p. 8491-8499.
10. Schaad, N.W., et al., *Xylella fastidiosa subspecies: X. fastidiosa subsp piercei, subsp. nov., X. fastidiosa subsp. multiplex subsp. nov., and X. fastidiosa subsp. pauca subsp. nov.* Systematic and applied microbiology, 2004. **27**(3): p. 290.
11. Almeida, R.P. and L. Nunney, *How Do Plant Diseases Caused by Xylella fastidiosa Emerge?* Plant Disease, 2015.
12. Cariddi, C., et al., *Isolation of a Xylella fastidiosa strain infecting olive and oleander in Apulia, Italy.* Journal of Plant Pathology, 2014. **96**(2): p. 425-429.
13. Bae, C., et al., *Infection processes of xylem-colonizing pathogenic bacteria: possible explanations for the scarcity of qualitative disease resistance genes against them in crops.* Theoretical and Applied Genetics, 2015. **128**(7): p. 1219-1229.
14. Meng, Y., et al., *Upstream migration of Xylella fastidiosa via pilus-driven twitching motility.* Journal of bacteriology, 2005. **187**(16): p. 5560-5567.
15. Mattick, J.S., *Type IV pili and twitching motility.* Annual Reviews in Microbiology, 2002. **56**(1): p. 289-314.
16. Tyree, M.T. and M.H. Zimmermann, *Xylem structure and the ascent of sap*2013: Springer Science & Business Media.
17. Pérez-Donoso, A.G., et al., *Cell wall-degrading enzymes enlarge the pore size of intervessel pit membranes in healthy and Xylella fastidiosa-infected grapevines.* Plant physiology, 2010. **152**(3): p. 1748-1759.
18. Baccari, C. and S. Lindow, *Assessment of the process of movement of Xylella fastidiosa within susceptible and resistant grape cultivars.* Phytopathology, 2011. **101**(1): p. 77-84.
19. Almeida, R.P., et al., *Use of a Green Fluorescent Strain for Analysis of Xylella fastidiosa Colonization of Vitis vinifera.* Applied and Environmental Microbiology, 2003. **69**(12): p. 7319-7327.

20. Sun, Q., et al., *Vascular occlusions in grapevines with Pierce's disease make disease symptom development worse.* Plant physiology, 2013. **161**(3): p. 1529-1541.
21. Retchless, A.C., et al., *Genomic insights into Xylella fastidiosa interactions with plant and insect hosts*, in *Genomics of Plant-Associated Bacteria*2014, Springer. p. 177-202.
22. De La Fuente, L., et al., *Assessing adhesion forces of type I and type IV pili of Xylella fastidiosa bacteria by use of a microfluidic flow chamber.* Applied and Environmental Microbiology, 2007. **73**(8): p. 2690-2696.
23. Voegel, T.M., et al., *Localization and characterization of Xylella fastidiosa haemagglutinin adhesins.* Microbiology, 2010. **156**(7): p. 2172-2179.
24. Guilhabert, M.R. and B.C. Kirkpatrick, *Identification of Xylella fastidiosa antivirulence genes: hemagglutinin adhesins contribute to X. fastidiosa biofilm maturation and colonization and attenuate virulence.* Molecular Plant-Microbe Interactions, 2005. **18**(8): p. 856-868.
25. Castiblanco, L.F. and G.W. Sundin, *New insights on molecular regulation of biofilm formation in plant-associated bacteria.* Journal of integrative plant biology, 2015.
26. de Souza, A.A., et al., *Gene expression profile of the plant pathogen Xylella fastidiosa during biofilm formation in vitro.* FEMS Microbiology Letters, 2004. **237**(2): p. 341-353.
27. Stoodley, P., et al., *Biofilms as complex differentiated communities.* Annual Reviews in Microbiology, 2002. **56**(1): p. 187-209.
28. Killiny, N., et al., *The exopolysaccharide of Xylella fastidiosa is essential for biofilm formation, plant virulence, and vector transmission.* Molecular Plant-Microbe Interactions, 2013. **26**(9): p. 1044-1053.
29. Chatterjee, S., et al., *Role of cyclic di-GMP in Xylella fastidiosa biofilm formation, plant virulence, and insect transmission.* Molecular Plant-Microbe Interactions, 2010. **23**(10): p. 1356-1363.
30. Matsumoto, A., et al., *XatA, an AT-1 autotransporter important for the virulence of Xylella fastidiosa Temecula1.* Microbiologyopen, 2012. **1**(1): p. 33-45.
31. Reddy, J.D., et al., *TolC is required for pathogenicity of Xylella fastidiosa in Vitis vinifera grapevines.* Molecular Plant-Microbe Interactions, 2007. **20**(4): p. 403-410.
32. Nascimento, R., et al., *The Type II Secreted Lipase/Esterase LesA is a Key Virulence Factor Required for Xylella fastidiosa Pathogenesis in Grapevines.* Scientific reports, 2016. **6**.
33. Voegel, T.M., et al., *Identification of a response regulator involved in surface attachment, cell–cell aggregation, exopolysaccharide production and virulence in the plant pathogen Xylella fastidiosa.* Molecular plant pathology, 2013. **14**(3): p. 256-264.
34. Cursino, L., et al., *Twitching motility and biofilm formation are associated with tonB1 in Xylella fastidiosa.* FEMS Microbiology Letters, 2009. **299**(2): p. 193-199.
35. Hennigar, S.R. and J.P. McClung, *Nutritional Immunity Starving Pathogens of Trace Minerals.* American Journal of Lifestyle Medicine, 2016. **10**(3): p. 170-173.
36. Ehrlich, H., *Microbes and metals.* Applied microbiology and biotechnology, 1997. **48**(6): p. 687-692.
37. Cobine, P.A., et al., *Xylella fastidiosa differentially accumulates mineral elements in biofilm and planktonic cells.* PloS one, 2013. **8**(1): p. e54936.
38. Andersen, P.C., et al., *Influence of xylem fluid chemistry on planktonic growth, biofilm formation and aggregation of Xylella fastidiosa.* FEMS Microbiology Letters, 2007. **274**(2): p. 210-217.

39. Festa, R.A. and D.J. Thiele, *Copper at the front line of the host-pathogen battle.* PLoS pathogens, 2012. **8**(9).
40. Rodrigues, C.M., et al., *Copper resistance of biofilm cells of the plant pathogen Xylella fastidiosa.* Applied microbiology and biotechnology, 2008. **77**(5): p. 1145-1157.
41. Navarrete, F. and L. De La Fuente, *Response of Xylella fastidiosa to zinc: decreased culturability, increased exopolysaccharide production, and formation of resilient biofilms under flow conditions.* Applied and Environmental Microbiology, 2014. **80**(3): p. 1097-1107.
42. Navarrete, F. and L. De La Fuente, *Zinc detoxification is required for full virulence and modification of the host leaf ionome by Xylella fastidiosa.* Molecular Plant-Microbe Interactions, 2015. **28**(4): p. 497-507.
43. Hood, M.I. and E.P. Skaar, *Nutritional immunity: transition metals at the pathogen–host interface.* Nature Reviews Microbiology, 2012. **10**(8): p. 525-537.
44. Silva-Stenico, M.E., et al., *Growth and siderophore production of Xylella fastidiosa under iron-limited conditions.* Microbiological research, 2005. **160**(4): p. 429-436.
45. Zaini, P.A., et al., *The iron stimulon of Xylella fastidiosa includes genes for type IV pilus and colicin V-like bacteriocins.* Journal of bacteriology, 2008. **190**(7): p. 2368-2378.
46. Domínguez, D.C., M. Guragain, and M. Patrauchan, *Calcium binding proteins and calcium signaling in prokaryotes.* Cell calcium, 2015. **57**(3): p. 151-165.
47. Parker, J.K., et al., *Calcium transcriptionally regulates the biofilm machinery of Xylella fastidiosa to promote continued biofilm development in batch cultures.* Environmental microbiology, 2016.
48. Lefèvre, C.T., et al., *Calcium ion-mediated assembly and function of glycosylated flagellar sheath of marine magnetotactic bacterium.* Molecular microbiology, 2010. **78**(5): p. 1304-1312.
49. Gode-Potratz, C.J., D.M. Chodur, and L.L. McCarter, *Calcium and iron regulate swarming and type III secretion in Vibrio parahaemolyticus.* Journal of bacteriology, 2010. **192**(22): p. 6025-6038.
50. Orans, J., et al., *Crystal structure analysis reveals Pseudomonas PilY1 as an essential calcium-dependent regulator of bacterial surface motility.* Proceedings of the National Academy of Sciences, 2010. **107**(3): p. 1065-1070.
51. Porsch, E.A., et al., *Calcium binding properties of the Kingella kingae PilC1 and PilC2 proteins have differential effects on type IV pilus-mediated adherence and twitching motility.* Journal of bacteriology, 2013. **195**(4): p. 886-895.
52. Patrauchan, M., et al., *Calcium influences cellular and extracellular product formation during biofilm-associated growth of a marine Pseudoalteromonas sp.* Microbiology, 2005. **151**(9): p. 2885-2897.
53. Sarkisova, S., et al., *Calcium-induced virulence factors associated with the extracellular matrix of mucoid Pseudomonas aeruginosa biofilms.* Journal of bacteriology, 2005. **187**(13): p. 4327-4337.
54. Mangwani, N., et al., *Calcium-mediated modulation of Pseudomonas mendocina NR802 biofilm influences the phenanthrene degradation.* Colloids and Surfaces B: Biointerfaces, 2014. **114**: p. 301-309.
55. Bilecen, K. and F.H. Yildiz, *Identification of a calcium-controlled negative regulatory system affecting Vibrio cholerae biofilm formation.* Environmental microbiology, 2009. **11**(8): p. 2015-2029.

56. Shukla, S.K. and T.S. Rao, *Effect of calcium on Staphylococcus aureus biofilm architecture: a confocal laser scanning microscopic study.* Colloids and Surfaces B: Biointerfaces, 2013. **103**: p. 448-454.
57. Arrizubieta, M.J., et al., *Calcium inhibits bap-dependent multicellular behavior in Staphylococcus aureus.* Journal of bacteriology, 2004. **186**(22): p. 7490-7498.
58. De La Fuente, L., et al., *The bacterial pathogen Xylella fastidiosa affects the leaf ionome of plant hosts during infection.* PloS one, 2013. **8**(5): p. e62945.
59. Leite, B., et al., *Genomics and X-ray microanalysis indicate that Ca^{2+} and thiols mediate the aggregation and adhesion of Xylella fastidiosa.* Brazilian Journal of Medical and Biological Research, 2002. **35**(6): p. 645-650.
60. Cruz, L.F., P.A. Cobine, and L. De La Fuente, *Calcium increases Xylella fastidiosa surface attachment, biofilm formation, and twitching motility.* Applied and Environmental Microbiology, 2012. **78**(5): p. 1321-1331.
61. Cruz, L.F., et al., *Calcium-enhanced twitching motility in Xylella fastidiosa is linked to a single PilY1 homolog.* Applied and Environmental Microbiology, 2014. **80**(23): p. 7176-7185.
62. Dominguez, D.C., *Calcium signalling in bacteria.* Molecular microbiology, 2004. **54**(2): p. 291-297.
63. Zampese, E. and P. Pizzo, *Intracellular organelles in the saga of Ca^{2+} homeostasis: different molecules for different purposes?* Cellular and Molecular Life Sciences, 2012. **69**(7): p. 1077-1104.
64. Hashimoto, K. and J. Kudla, *Calcium decoding mechanisms in plants.* Biochimie, 2011. **93**(12): p. 2054-2059.
65. Jones, H.E., et al., *Slow changes in cytosolic free Ca^{2+} inEscherichia colihighlight two putative influx mechanisms in response to changes in extracellular calcium.* Cell calcium, 1999. **25**(3): p. 265-274.
66. Torrecilla, I., et al., *Use of recombinant aequorin to study calcium homeostasis and monitor calcium transients in response to heat and cold shock in cyanobacteria.* Plant physiology, 2000. **123**(1): p. 161-176.
67. Nazarenko, L.V., et al., *Calcium release from Synechocystis cells induced by depolarization of the plasma membrane: MscL as an outward Ca^{2+} channel.* Microbiology, 2003. **149**(5): p. 1147-1153.
68. Chang, Y., et al., *Structural basis for a pH-sensitive calcium leak across membranes.* Science, 2014. **344**(6188): p. 1131-1135.
69. Palmgren, M.G. and K.B. Axelsen, *Evolution of P-type ATPases.* Biochimica et Biophysica Acta (BBA)-Bioenergetics, 1998. **1365**(1): p. 37-45.
70. Sarkisova, S.A., et al., *A Pseudomonas aeruginosa EF-hand protein, EfhP (PA4107), modulates stress responses and virulence at high calcium concentration.* PloS one, 2014. **9**(6): p. e98985.
71. Domníguez, D.C., et al., *Proteome Analysis of B. subtilis in Response to Calcium.* Journal of Analytical & Bioanalytical Techniques, 2013. **2011**.
72. Jones, H., I. Holland, and A. Campbell, *Direct measurement of free Ca^{2+} shows different regulation of Ca^{2+} between the periplasm and the cytosol of Escherichia coli.* Cell calcium, 2002. **32**(4): p. 183-192.

73. Seufferheld, M., et al., *The H^+-pyrophosphatase of Rhodospirillum rubrum is predominantly located in polyphosphate-rich acidocalcisomes.* Journal of Biological Chemistry, 2004. **279**(49): p. 51193-51202.
74. Rigden, D.J. and M.Y. Galperin, *The DxDxDG motif for calcium binding: multiple structural contexts and implications for evolution.* Journal of molecular biology, 2004. **343**(4): p. 971-984.
75. Johnson, M.D., et al., *Pseudomonas aeruginosa PilY1 binds integrin in an RGD-and calcium-dependent manner.* PloS one, 2011. **6**(12): p. e29629.
76. Wang, S.-L., et al., *CabC, an EF-hand calcium-binding protein, is involved in Ca2+-mediated regulation of spore germination and aerial hypha formation in Streptomyces coelicolor.* Journal of bacteriology, 2008. **190**(11): p. 4061-4068.
77. Zhao, Y., et al., *CcbP, a calcium-binding protein from Anabaena sp. PCC 7120, provides evidence that calcium ions regulate heterocyst differentiation.* Proceedings of the National Academy of Sciences of the United States of America, 2005. **102**(16): p. 5744-5748.
78. Prost, L.R. and S.I. Miller, *The Salmonellae PhoQ sensor: mechanisms of detection of phagosome signals.* Cellular microbiology, 2008. **10**(3): p. 576-582.
79. Kwon, H.Y., et al., *Ca^{2+}-dependent expression of the CIRCE regulon in Streptococcus pneumoniae.* Molecular microbiology, 2005. **55**(2): p. 456-468.
80. Patrauchan, M.A., S.A. Sarkisova, and M.J. Franklin, *Strain-specific proteome responses of Pseudomonas aeruginosa to biofilm-associated growth and to calcium.* Microbiology, 2007. **153**(11): p. 3838-3851.
81. Fjellbirkeland, A., et al., *Molecular analysis of an outer membrane protein, MopB, of Methylococcus capsulatus (Bath) and structural comparisons with proteins of the OmpA family.* Archives of microbiology, 2000. **173**(5-6): p. 346-351.
82. Chen, Y.-Y., et al., *Mutation of the gene encoding a major outer-membrane protein in Xanthomonas campestris pv. campestris causes pleiotropic effects, including loss of pathogenicity.* Microbiology, 2010. **156**(9): p. 2842-2854.
83. Dandekar, A.M., et al., *An engineered innate immune defense protects grapevines from Pierce disease.* Proceedings of the National Academy of Sciences, 2012. **109**(10): p. 3721-3725.
84. Koebnik, R., K.P. Locher, and P. Van Gelder, *Structure and function of bacterial outer membrane proteins: barrels in a nutshell.* Molecular microbiology, 2000. **37**(2): p. 239-253.
85. El Hassouni, M., et al., *The minimal gene set member msrA, encoding peptide methionine sulfoxide reductase, is a virulence determinant of the plant pathogen Erwinia chrysanthemi.* Proceedings of the National Academy of Sciences, 1999. **96**(3): p. 887-892.
86. Weissbach, H., L. Resnick, and N. Brot, *Methionine sulfoxide reductases: history and cellular role in protecting against oxidative damage.* Biochimica et Biophysica Acta (BBA)-Proteins and Proteomics, 2005. **1703**(2): p. 203-212.
87. Dhandayuthapani, S., et al., *Peptide methionine sulfoxide reductase (MsrA) is a virulence determinant in Mycoplasma genitalium.* Journal of bacteriology, 2001. **183**(19): p. 5645-5650.

88. Douglas, T., et al., *Methionine sulfoxide reductase A (MsrA) deficiency affects the survival of Mycobacterium smegmatis within macrophages.* Journal of bacteriology, 2004. **186**(11): p. 3590-3598.
89. Wizemann, T.M., et al., *Peptide methionine sulfoxide reductase contributes to the maintenance of adhesins in three major pathogens.* Proceedings of the National Academy of Sciences, 1996. **93**(15): p. 7985-7990.

Chapter 2:
Calcium prolongs biofilm development of *Xylella fastidiosa* strain 'Temecula'

Abstract

Pierce's disease of grapevine, a disease caused by *Xylella fastidiosa*, causes millions of dollars in losses every year in southern areas of the United States. Symptom development is associated with blockage of water transport in the xylem network of grapevine, caused by extensive colonization of this pathogen. The mineral content of the xylem modulates bacterial growth and virulence. Research conducted in our laboratory has proven that calcium (Ca) increases biofilm formation of *X. fastidiosa* by affecting the initial stages of biofilm development. However, the effect of calcium on the later stages of biofilm development has not been characterized. In this present study, biofilm and planktonic growth of *X. fastidiosa* 'Temecula' over time cultured in different Ca-amended media was quantified. Results show that when cells are grown in non-Ca amended medium (0.02mM Ca), by 96h they have already reached the dispersion stage, while cells in Ca-supplemented medium (4 mM) are still forming dense, mature biofilms over the same time period. These results indicate that Ca supplementation contributes to prolong biofilm development of *X. fastidiosa* strain 'Temecula'.

Introduction

Pierce's disease, an important disease on grapevines, is a constant threat to the wine industry of the United States, causing millions of dollars in losses every year in California [1] and other states in southern areas of the country. *Xylella fastidiosa* subsp. *fastidiosa* strain 'Temecula' is the type strain used to study this disease[2].

The widely accepted explanation for the pathogenicity of *X. fastidiosa* is that it causes occlusion of the xylem network of grapevine which impairs the water and mineral nutrient transport from the roots to the leaves of the plant [3]. The occlusion is caused by aggregated cells forming biofilms, as well as tyloses formed by grapevines in response to *X. fastidiosa* infection [4, 5].

Biofilm formation has been extensively studied in many bacterial species, since it is an important adaptation mechanism affecting survival of bacteria in most habitats. Biofilm protects bacterial cells from harsh environmental factors and promotes nutrient uptake [6]. In general, biofilm formation consists of five stages including: 1) reversible adhesion, 2) irreversible adhesion in conjunction with secretion of exopolymeric substances and loss of motility, 3) early development of biofilm architecture, 4) development of mature biofilms with complex architecture, 5) dispersion of motile cells in a planktonic state [6-8].

In plants, *X. fastidiosa* is restricted to live in the xylem system, an environment that is poor in nutrients [6]. Biofilm formed by this pathogen are vital for its persistence in this environment [6]. Meanwhile, the content of the xylem regulate the biofilm formation of *X. fastidiosa*. Xylem is the conduit for the transportation of mineral nutrient uptake by the plant roots. Mineral elements, such as iron (Fe), zinc (Zn), copper (Cu), and manganese (Mn) are required for almost every critical biological process in both host and pathogen, and are essential at the host-pathogen interface [9, 10]. Previous study conducted by our laboratory shows that Ca promote surface attachment, biofilm formation and twitching motility of *X. fastidiosa in vitro* [11]. That study demonstrated that Ca affects the initial stages of biofilm development including surface attachment and cell aggregation. However, the role of Ca on the whole process of biofilm

formation over time is still unclear; in particular, the effect of Ca on the dispersion of biofilm at later stages. In this study, the biofilm and planktonic growth of *X. fastidiosa* cultured in a basal undefined medium (PD2), and PD2 supplemented with Ca at a concentration similar to what is found in grapevine xylem sap (4 mM) [12], was quantified over time. The results presented here indicate that Ca delays the dispersion of biofilm. Calcium supplementation contributes to prolong biofilm development of *X. fastidiosa* strain 'Temecula' over a period of time tested. This information is important to understand the disease progression of *X. fastidiosa* in plants, in particular inside the xylem network where Ca is transported.

Materials and methods

Bacterial strains and culture conditions.

Xylella fastidiosa strain 'Temecula' was grown on PW [13] agar plates at 28°C for 7 days. The culture was scraped from the plates, resuspended in PD2 [14] liquid medium, and adjusted to OD_{600} = 0.1. Autoclaved 250-ml glass flasks with foam stoppers were filled with 50 ml liquid culture medium, half with PD2 ("PD2" treatment) and half with PD2 supplemented with 4 mM $CaCl_2$ ("4Ca" treatment). Each flask was inoculated with 1 ml bacterial suspension and incubated at 28°C with shaking at 150 rpm.

Biofilm and planktonic growth quantification.

X. fastidiosa was cultured in 26 250-ml glass flasks as described above, half with PD2 and half with 4Ca. Every 24 hours after inoculation, for a total of four time points ("T1", "T2", "T3", and "T4"), biofilm formed at the air-liquid interface was harvested from triplicate flasks of each

treatment, resuspended in 20 ml PD2 or 4Ca depending on the treatment, and thoroughly homogenized by pipetting. OD_{600} of six 200 µl aliquots per biofilm suspension was measured. In addition, two 200 µl aliquots per biofilm suspension were used directly for DNA extraction using a modified cetyltrimethylammonium bromide (CTAB) method [15], and two 200 µl aliquots were treated with ethidium monoazide (EMA) as described previously [16] and subsequently used for DNA extraction. In total, this yielded six replicates per sample type per treatment per time. For the planktonic phase, 40 ml liquid culture were collected from single flasks of each treatment upon inoculation ("T0") as well as from the same triplicate flasks of each treatment used for biofilm sample collection (time points T1, T2, T3, and T4). All planktonic samples were treated in the same manner as the biofilm suspensions. Two independent experiments were performed.

Quantification of *X. fastidiosa* cells in biofilm and planktonic phases by qPCR.

Sample concentrations of total and viable *X. fastidiosa* cells were determined by quantitative PCR (qPCR) of the non-EMA-treated and EMA-treated DNA samples, respectively, as previously described [16]. qPCR reactions (20 µl) consisted of 4 µl PerfeCTa Multiplex qPCR ToughMix Low Rox (Quanta Biosciences), 0.4 µM HLP TaqMan probe, 0.2 µM each primer HL5/HL6 [17], and 1 µl DNA. qPCR was performed on a CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories) with the following cycling parameters: 95 ℃ for 8 min, followed by 45 cycles of 95 ℃ for 15 s and 60 ℃ for 1 min. A four-point standard curve of 10-fold serially diluted *X. fastidiosa* DNA was amplified alongside each set of samples for CFU quantification as previously described [18].

Statistical analyses

For the quantification of biofilm and planktonic growth, data were analyzed by SAS 9.4 (SAS Institute Inc.). To compare suspension concentration differences between the two treatments at each time point, OD_{600} data were evaluated by t-test ($P < 0.05$). Total and viable *X. fastidiosa* cell concentrations in biofilm and planktonic fractions were analyzed by the GLIMMIX procedure. Means were separated by the Tukey-Kramer method ($P < 0.05$).

Results

***X. fastidiosa* 'Temecula' biofilm and planktonic growth.**

Faint biofilm growth was visible at the air-liquid interface of the culture flasks at T1 (24 hrs), and growth increased over time through T4 (96 hrs) (Fig. 1A). Compared to biofilm cultured in PD2 liquid medium [19] ("PD2" treatment), biofilm growth in PD2 supplemented with 4 mM $CaCl_2$ ("4Ca" treatment) was greater and denser at all 24-hr time points (T1-T4) (Fig. 1A). Biofilm in PD2 never achieved the appearance of that in 4Ca, and at later time points (T3, T4) sections of the biofilm had detached from the glass, conferring a patchy appearance (Fig. 1A and 1B). By 96 hrs, a significant portion of the biofilm in PD2 had detached, while the majority of biofilm in 4Ca was still attached. Observations of the flasks bottoms (Fig. 1B) revealed that precipitates were accumulating in PD2 at T3 and T4, while these were barely visible in 4Ca. Quantification of the OD_{600} (Fig. 2) corroborated these visual observations and indicated that significantly higher biofilm (Fig. 2A) was detected in T3 ($P = 0.004$) and T4 ($P < 0.0001$) in 4Ca compared to PD2. The opposite trend was observed for planktonic growth (Fig. 2B), which was significantly higher ($P < 0.001$) for PD2 in T3 and T4, probably due to biofilm aggregates falling from the flask walls into the liquid suspension (Fig. 1).

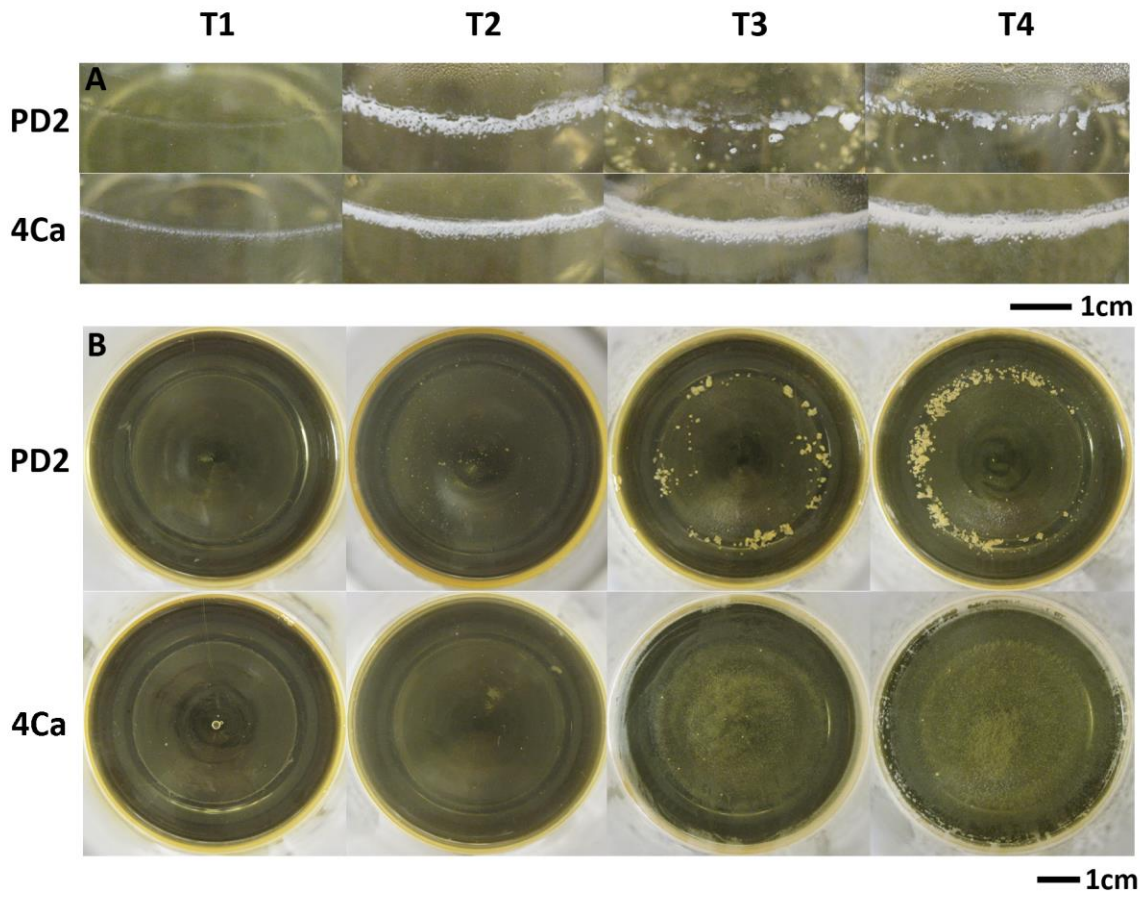

Figure 2-1. *X. fastidiosa* growth after 24 (T1), 48 (T2), 72 (T3), and 96 (T4) hrs in glass flasks containing PD2 media (PD2) or PD2 media supplemented with 4 mM $CaCl_2$ (4Ca). (A) Images of biofilm growth on flask walls. (B) Images of the bottoms of the glass flasks to show aggregate precipitation in the liquid media (planktonic growth). Scale bars represent 1 cm.

When viable biofilm cells were quantified (Fig. 3A), 4Ca had significantly higher concentrations than PD2 at all time points (P = 0.002, 0.02, 0.0009, 0.0003 for T1-T4, respectively). For viable planktonic cells (Fig. 3B), significantly higher concentrations were detected at T1 (P = 0.0014) and T4 (P < 0.0001) in PD2 compared to 4Ca, while concentrations were higher in 4Ca at T0 (P = 0.001). When total cells were quantified (Fig. 3C and 3D), significantly higher concentrations

were detected in biofilm growth in 4Ca compared to PD2 at T3 (P = 0.046) and T4 (P =0.035), while in planktonic growth higher concentrations of cells were detected in PD2 at T2, T3, and T4 (P= 0.0015, 0.0007, 0.0008, respectively) (Fig. 3D) compared to 4Ca.

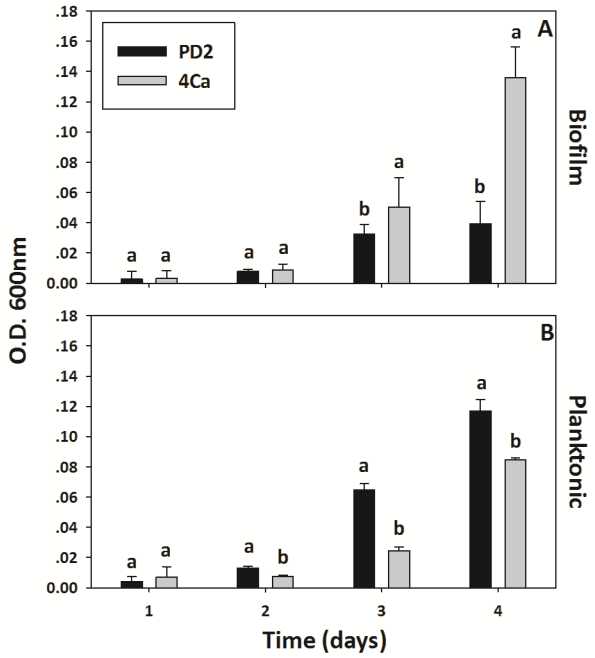

Figure 2-2. Quantification by OD_{600} of *X. fastidiosa* biofilm formation (A) and planktonic growth (B) at different time points. Each data point represents means (n = 18) from one representative experiment; error bars correspond to standard deviation (SD) of the mean. Different letters indicate significant differences (P < 0.05) between PD2 and 4Ca treatments at each time point according to *t* test.

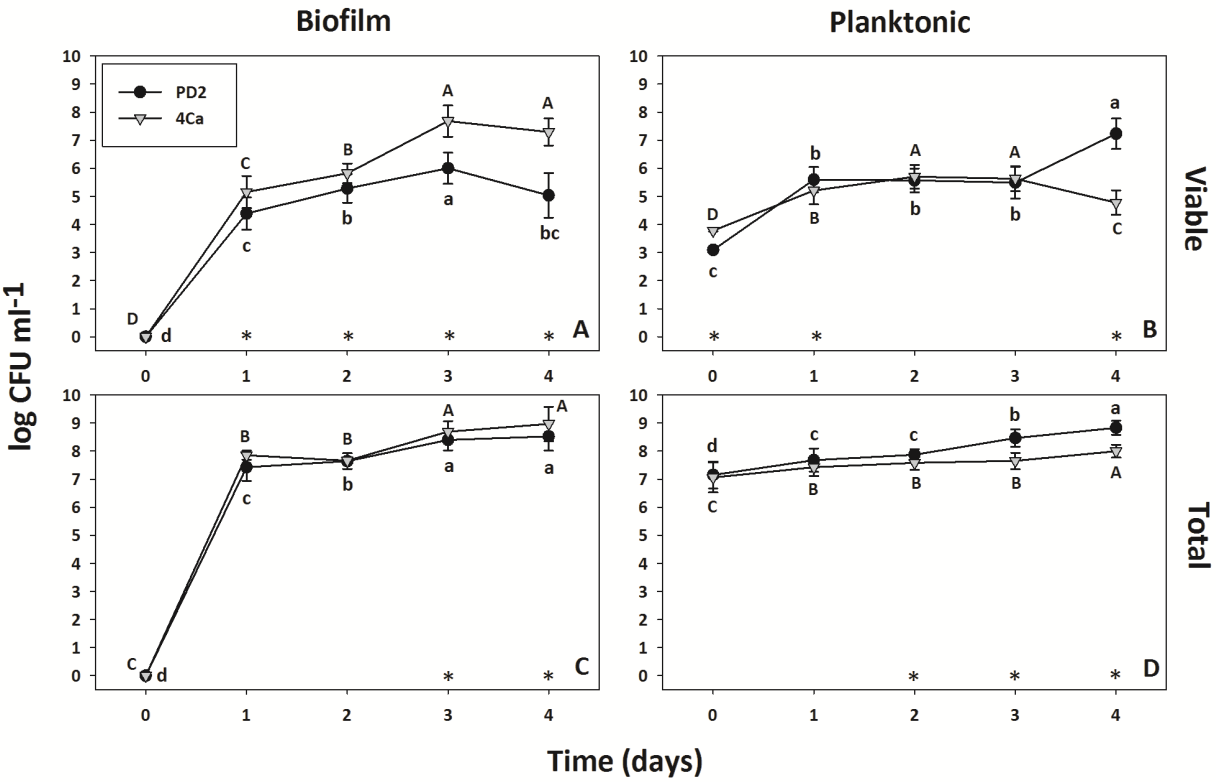

Figure 2-3. Growth curves of *X. fastidiosa* in PD2 and 4Ca. The concentration of viable (A and B) and total (C and D) cells in biofilm and planktonic phases at different time points was determined by qPCR. Each data point represents the mean ± SD (n = 6) from one representative experiment. For each treatment in the same graph, different letters indicate statistical differences ($P < 0.05$) between each time point. Lowercase and uppercase letters correspond to PD2 and 4Ca treatments, respectively. Asterisk indicates a significant difference ($P < 0.05$) between PD2 and 4Ca at each time point. Analysis was performed by the GLIMMIX procedure.

Discussion

Calcium as an essential mineral nutrient for living organisms that impacts many critical biological processes [20]. The effect of Ca on biofilm has been studied in plant-associated and animal-associated bacteria [11, 21]. For example, in *Pseudomonas aeruginosa*, an opportunistic

pathogen for humans, Ca increases the mechanical strength and thickness of the biofilm formed by this pathogen [21, 22]. On the contrary, increase of Ca concentrations reduces the thickness of biofilm formed by *Staphylococcus aureus* Strain V329 [23].

In *X. fastidiosa,* Ca plays a key role for cell attachment at the initial stages of biofilm development [11]. However, the impact of Ca on the whole process of biofilm formation, especially on the later stages, was unclear. In the present study, the growth of *X. fastidiosa* strain 'Temecula' in PD2 treatment and 4Ca treatment has been monitored for 96 hours. In addition, turbidity and cells numbers of biofilm and planktonic phases have been quantified. Based on these results, it was estimated that different time points represent different stages in the process of biofilm formation. For PD2 treatment, 24 hours (T1) represents the stage 2 (irreversible adhesion and aggregation) and/or 3 (early maturation), 48 hours (T2) represents stage 4 (mature biofilms), 72 and 96 hours (T3 and T4) represent stage 5 (dispersion). For 4Ca treatment, 24 hours (T1) represents the stage 3 (early maturation), 48, 72 and 96 hours (T2, T3 and T4) represents stage 4 (mature biofilms). It indicated that Ca inhibits *X. fastidiosa* cell detachment from biofilm into planktonic phase and prolong mature biofilm phase.

Previous research offered possible explanations aimed at understanding how Ca impacts the architecture and machinery of biofilm. Ca as a divalent cation have been implicated as a potent crosslinker in the biofilm formation process [24]. Leite et al. [25] proposed a model of Ca mediating adhesion and aggregation of *X. fastidiosa*: Ca and Mg work as divalent cation bridges connecting negatively charged thiol groups on bacterial surface and negatively charged xylem vessels. More recently, a study has shown that Ca act as cationic bridging agent, connecting

extracellular DNA on the bacterial cells surface, to impact bacterial cell aggregation and biofilm formation [26]. Besides this physical effect, Ca act as an important signal molecule in cells, also regulating expression of genes related to biofilm formation [20]. When *X. fastidiosa* was treated by tetracycline, which inhibits bacterial de novo protein synthesis, cells did not increase adhesion force in Ca supplemented medium [11]. This indicates that Ca has a regulatory role in cell adhesion [11, 27]. In *P. aeruginosa*, Ca addition increased the production of alginate and three extracellular enzymes, which are important components of the extracellular matrix of *P. aeruginosa* biofilm [21]. In addition, Ca altered global proteome profiles of *P. aeruginosa* [28], *Pseudoalteromonas* sp. 1398 [29], and *Citrobacter werkmanii* BF-6 [30] biofilm, which suggested that Ca can modulates the expression of hundreds of genes.

Ca enhanced biofilm formation and prolonged biofilm development of *X. fastidiosa* is not only caused by its cationic bridging effect, but also induced by its regulatory role. Recently, comparative transcriptome analyses by RNA-Seq of *X. fastidiosa* biofilm cells grown in different Ca supplemented and non-supplemented media were conducted in our laboratory. The results indicated that more than two hundred genes were transcriptionally regulated by external Ca. These genes were associated with adhesion, peptidoglycan synthesis/recycling, motility, virulence, metal homeostasis, two-component systems and others functions [27]. In this study, when cells in PD2 have already reached the dispersion stage, cells in 4Ca are still forming mature biofilms. These observations are supported by the results of the comparative transcriptome analysis [27], where expression patterns for genes related with fimbrial and afimbrial adhesins are indicators of the differences of biofilm formation in PD2 and 4Ca. Genes including *pilA*, *pilH* and *pilT*, which are associated with the function of type IV pili contributing

the twitching motility, were expressed at high levels in PD2 treatments at 72 hours after inoculation, but were expressed at low levels in 4Ca treatments at the same time [27]. In contrast, genes including *pspA*, *xadA*, *hxfA* and *hsf*, which encode afimbrial adhesins, were expressed at low levels in PD2 treatments but at high level in 4Ca treatments at 72 hours [27]. In addition, nine genes related with peptidoglycan biosynthesis and recycling were upregulated in 4Ca treatment [27]. These expression patterns suggested that at the later stages of biofilm development, cells in PD2 treatment become more motile, but cells in 4Ca treatment still continue the aggregation process. Though the transcriptome analysis confirms the regulatory role of Ca in biofilm formation of *X. fastidiosa*, the exact mechanisms and regulatory pathway related with the process is still unclear and further research is necessary.

This study demonstrated that Ca supplementation contributes to prolong biofilm development of *X. fastidiosa* strain 'Temecula'. Xylem sap of host plant is the living environment and the source of Ca and other mineral nutrients for this pathogen. In grapevines, this pathogen is exposed to an average sap Ca concentration of 4mM [12], which is the same used for the 4Ca treatment in this study. This Ca concentration can prolong biofilm formation that will cause more xylem occlusions, leading to more severe symptoms. Overall, the study presented here is important to understand the disease progression of *X. fastidiosa* in plants. In addition, further understanding of the response of *X. fastidiosa* to Ca will be critical to explore novel targets for disease management applications.

References

1. Tumber, K.P., J.M. Alston, and K. Fuller, *Pierce's disease costs California $104 million per year.* California Agriculture, 2014. **68**(1-2).
2. Van Sluys, M., et al., *Comparative analyses of the complete genome sequences of Pierce's disease and citrus variegated chlorosis strains of Xylella fastidiosa.* Journal of bacteriology, 2003. **185**(3): p. 1018-1026.
3. Chatterjee, S., R.P. Almeida, and S. Lindow, *Living in two worlds: the plant and insect lifestyles of Xylella fastidiosa.* Phytopathology, 2008. **46**(1): p. 243.
4. Almeida, R.P., et al., *Use of a Green Fluorescent Strain for Analysis of Xylella fastidiosa Colonization of Vitis vinifera.* Applied and Environmental Microbiology, 2003. **69**(12): p. 7319-7327.
5. Sun, Q., et al., *Vascular occlusions in grapevines with Pierce's disease make disease symptom development worse.* Plant physiology, 2013. **161**(3): p. 1529-1541.
6. Castiblanco, L.F. and G.W. Sundin, *New insights on molecular regulation of biofilm formation in plant‐associated bacteria.* Journal of integrative plant biology, 2015.
7. Sauer, K., et al., *Pseudomonas aeruginosa displays multiple phenotypes during development as a biofilm.* Journal of bacteriology, 2002. **184**(4): p. 1140-1154.
8. Stoodley, P., et al., *Biofilms as complex differentiated communities.* Annual Reviews in Microbiology, 2002. **56**(1): p. 187-209.
9. Hood, M.I. and E.P. Skaar, *Nutritional immunity: transition metals at the pathogen–host interface.* Nature Reviews Microbiology, 2012. **10**(8): p. 525-537.
10. Hänsch, R. and R.R. Mendel, *Physiological functions of mineral micronutrients (Cu, Zn, Mn, Fe, Ni, Mo, B, Cl).* Current opinion in plant biology, 2009. **12**(3): p. 259-266.
11. Cruz, L.F., P.A. Cobine, and L. De La Fuente, *Calcium increases Xylella fastidiosa surface attachment, biofilm formation, and twitching motility.* Applied and Environmental Microbiology, 2012. **78**(5): p. 1321-1331.
12. Cobine, P.A., et al., *Xylella fastidiosa differentially accumulates mineral elements in biofilm and planktonic cells.* PloS one, 2013. **8**(1): p. e54936.
13. Davis, M., A. Purcell, and S. Thomson, *Isolation media for the Pierce's disease bacterium.* Phytopathology, 1980. **70**(5): p. 425-429.
14. Davis, M.J., W.J. French, and N.W. Schaad, *Axenic culture of the bacteria associated with phony disease of peach and plum leaf scald.* Current Microbiology, 1981. **6**(5): p. 309-314.
15. Doyle, J. and J.L. Doyle, *Genomic plant DNA preparation from fresh tissue - CTAB method.* Phytochem. Bull., 1987. **19**: p. 11-15.
16. Navarrete, F. and L. De La Fuente, *Response of Xylella fastidiosa to zinc: decreased culturability, increased exopolysaccharide production, and formation of resilient biofilms under flow conditions.* Appl Environ Microbiol, 2014. **80**(3): p. 1097-107.
17. Francis, M., et al., *Genome-based PCR primers for specific and sensitive detection and quantification of Xylella fastidiosa.* European Journal of Plant Pathology, 2006. **115**(2): p. 203-213.
18. De La Fuente, L., et al., *The bacterial pathogen Xylella fastidiosa affects the leaf ionome of plant hosts during infection.* PLoS ONE, 2013. **8**(5): p. e62945.
19. Davis, M.J., A.H. Purcell, and S.V. Thomson, *Isolation media for the Pierce's disease bacterium.* Phytopathology, 1980. **70**(5): p. 425-429.

20. Domínguez, D.C., M. Guragain, and M. Patrauchan, *Calcium binding proteins and calcium signaling in prokaryotes.* Cell calcium, 2015. **57**(3): p. 151-165.
21. Sarkisova, S., et al., *Calcium-induced virulence factors associated with the extracellular matrix of mucoid Pseudomonas aeruginosa biofilms.* Journal of bacteriology, 2005. **187**(13): p. 4327-4337.
22. Körstgens, V., et al., *Influence of calcium ions on the mechanical properties of a model biofilm of mucoid Pseudomonas aeruginosa.* Water Science and Technology, 2001. **43**(6): p. 49-57.
23. Shukla, S.K. and T.S. Rao, *Effect of calcium on Staphylococcus aureus biofilm architecture: a confocal laser scanning microscopic study.* Colloids and Surfaces B: Biointerfaces, 2013. **103**: p. 448-454.
24. Chen, X. and P. Stewart, *Role of electrostatic interactions in cohesion of bacterial biofilms.* Applied microbiology and biotechnology, 2002. **59**(6): p. 718-720.
25. Leite, B., et al., *Genomics and X-ray microanalysis indicate that Ca^{2+} and thiols mediate the aggregation and adhesion of Xylella fastidiosa.* Brazilian Journal of Medical and Biological Research, 2002. **35**(6): p. 645-650.
26. Das, T., et al., *Influence of calcium in extracellular DNA mediated bacterial aggregation and biofilm formation.* PloS one, 2014. **9**(3): p. e91935.
27. Parker, J.K., et al., *Calcium transcriptionally regulates the biofilm machinery of Xylella fastidiosa to promote continued biofilm development in batch cultures.* Environmental microbiology, 2016.
28. Patrauchan, M.A., S.A. Sarkisova, and M.J. Franklin, *Strain-specific proteome responses of Pseudomonas aeruginosa to biofilm-associated growth and to calcium.* Microbiology, 2007. **153**(11): p. 3838-3851.
29. Patrauchan, M., et al., *Calcium influences cellular and extracellular product formation during biofilm-associated growth of a marine Pseudoalteromonas sp.* Microbiology, 2005. **151**(9): p. 2885-2897.
30. Zhou, G., et al., *Proteome responses of Citrobacter werkmanii BF-6 planktonic cells and biofilms to calcium chloride.* Journal of proteomics, 2015.

Chapter 3:
Virulence traits and disease development are impaired (*mopB*) or non-affected (*msrA*) in deletion mutants of two wild-type *Xylella fastidiosa* strains

Abstract

Xylella fastidiosa is a gram negative pathogenic bacterium that causes many economically important diseases including Pierce's disease in grapevine, citrus variegated chlorosis, and others. The development of disease symptom is associated with the blockage of water flow, caused by extensive colonization of the host xylem system by *X. fastidiosa*. Surface attachment, biofilm formation and twitching motility are critical virulence traits of *X. fastidiosa* that are affected by calcium (Ca), an important mineral nutrient in xylem sap. Here, we evaluated the role of two *X. fastidiosa* Ca-related genes (*mopB* and *msrA*) in virulence traits in vitro and disease development in vivo. *mopB* and *msrA* mutants in two *X. fastidiosa* strains 'Temecula' and 'WM1-1' were constructed by site-directed mutagenesis. All knockouts had similar response to Ca supplementation as the respective WT strains, indicating that these particular genes are not responsible for the increase in biofilm and/or movement observed in WT strains after Ca supplementation. Nevertheless, *mopB* mutants in both background strains were impaired in surface attachment, biofilm formation, and twitching motility. In addition, *mopB* mutants were impaired in pilus formation as observed by electron microscopy. *mopB* mutants in both backgrounds showed reduced virulence when tested on tobacco as a host under greenhouse conditions. In contrast, the *msrA* mutation in either background strain had no effect on virulence traits and disease development. These results suggest that outer membrane protein MopB is required for biofilm formation, motility, pilus biogenesis and virulence of *X. fastidiosa* but the *msrA* gene played no role in these processes under the conditions evaluated.

Introduction

Xylella fastidiosa is a gram negative pathogenic bacterium that causes many economically important disease including Pierce's disease in grapevine, citrus variegated chlorosis, and others [1]. The pathogen is transmitted by xylem sap-feeding insects, such as sharpshooters, leafhoppers, and spittlebugs through directly injecting the bacterium into xylem vessels [2, 3]. The bacterium strictly colonizes the xylem vessels [2], where upstream migration is important for colonization of the host. Upstream migration is mediated by type IV pilus-driven twitching motility [4]. In addition to type IV pili, *X. fastidiosa* has type I pili is associated with bacterial adhesion, and both pili are anchored on one pole of the bacterial cell [5]. The bacterial pathogen attaches to the walls of plant xylem and forms biofilms which inhibits water transportation in the xylem. The development of disease symptom is associated with the blockage of water flow [2, 3]. Overall, twitching motility and biofilm formation are critical virulence traits of *X. fastidiosa.*

Previous reports indicate that mineral elements in the xylem can affect traits associated with the virulence of *X. fastidiosa*. Iron (Fe), copper (Cu), zinc (Zn) and calcium (Ca) have been implicated in the infection process of this pathogen. Fe was found to affects growth and siderophore production by *X. fastidiosa*, and regulates gene expression related with the type IV pilus, and colicin V-like bacteriocins [6, 7]. According to *in vitro* experiments, Cu and Zn present in xylem fluid were correlated with the growth of *X. fastidiosa* [8]. In addition, Zn has been demonstrated as a stress factor with pleiotropic effects on *X. fastidiosa,* decreasing growth of the bacterium and biofilm formation, but increasing exopolysaccharide production and adhesion strength of biofilm [9]. In addition, Ca was found to increase cell attachment, biofilm

formation, and twitching motility of *X. fastidiosa* [10]. In chapter 2 of this thesis, I demonstrated that Ca supplementation prolongs the time period for biofilm formation. Ca was also found to accumulate at a higher concentration in biofilm than that in planktonic cells [11]. Among the mineral elements mentioned above, Ca has the most significant effect on the virulence traits of *X. fastidiosa*.

Calcium is a secondary messenger in eukaryotic cells and regulates many important cellular processes through Ca signal pathways and Ca binding proteins [12, 13]. Compared to the extensive studies of Ca in eukaryotes, the research of the role of Ca in prokaryotes still lags behind. However, during recent years, increasing evidence indicates that Ca plays a regulatory role in prokaryotes [14]. There are two main mechanisms by which Ca can influence protein activity and therefore bacterial metabolism: i) Ca can modulate gene expression in prokaryotes. Transcriptome analysis showed that Ca can negatively regulate the expression of genes involved in biofilm formation of the bacterium *Vibrio cholerae* [15]. In contrast, through comparing whole transcriptome of *X. fastidiosa* biofilm cells grown in different Ca supplemented and non-supplemented media, it was determined that Ca regulates the expression of genes involved in attachment, motility, and EPS biosynthesis to promote continued biofilm development [16]. Proteome analysis of *Bacillus subtilis* and *Pseudomonas aeruginosa* revealed that the expression of hundreds of genes are modulated by Ca supplementation [17, 18]. ii) Ca directly interacts with Ca-binding proteins to affect the physiology of prokaryotes. Recently, a number of Ca-binding proteins have been identified in prokaryotic organisms. Most of these proteins contain sequences closely resembling the typical eukaryotic Ca-binding motif, EF-hand motif [14]. Four conserved EF-hand motifs in bacteria have been reported: DxDxDG [19], Dx[DN]xDGxxD [20],

DxDxNxxxD [21], and Dx[DN]xDxxxxxx[DE] [22]. Ca can regulate the surface adhesion and motility of the bacteria *Kingella kingae*, *Pseudomonas aeruginosa* and *X. fastidiosa* through proteins containing EF-hand motifs [20, 22, 23]. By searching *X. fastidiosa* 'Temecula' genome, 42 genes were identified that contain one of the four Ca-biding motifs sequence patterns [16]. Seven of these genes were differently expressed dependent on Ca concentration in the media, according to whole transcriptome analysis [16]. However, experimental studies confirming that these proteins can bind to Ca are lacking. An exception is the study on the type IV pilus protein PilY1 of *X. fastidiosa* [23], whose Ca binding ability was inferred by homology and indirect evidence from biochemical studies.

A few studies have been focused on understanding the mechanism of Ca enhancement of virulence traits of *X. fastidiosa*. Ca is a divalent cation that can work as an ion bridge between bacterial cells, as well as cells and xylem surfaces, which contributes to the bacterial adhesion [24]. Besides a physical effect, a regulatory effect of Ca on *X. fastidiosa* virulence traits was found. Using tetracycline to disrupt *de novo* protein synthesis of *X. fastidiosa*, it was found that this pathogen failed to respond to Ca in terms of increased surface attachment [10]. Ca also interacts with a type IV pilus structural protein PilY1 which contains a Ca binding motif, enhancing twitching motility of *X. fastidiosa* [23]. Recently, comparative transcriptome analyses conducted by our group indicate that gene expression of biofilm cells are broadly modified by Ca supplementation [16]. These results confirm the regulatory role of Ca in *X. fastidiosa*, however, further studies are needed to fully understand the mechanism of Ca regulations of virulence traits on *X. fastidiosa*.

In this chapter, in order to provide information for further understanding of the mechanism of *X. fastidiosa* response to Ca, two putative Ca-related genes *mopB* and *msrA* were selected to focus our studies. i) *mopB* encodes the major outer membrane protein MopB. Based on DNA sequence analysis, the C-terminal part of MopB is a homologue of OmpA family; and a putative EF-hand motif, Dx[DN]xDxxxxxx[DE], is also located in this part of the protein. This protein is required for pathogenicity *of Xanthomonas campestris* pv. *campestris,* a phytopathogen that causes black rot in crucifers [25]. However, the role of MopB in virulence of *X. fastidiosa* has not been studied. Dandekar et al. [26] identified MopB as a recognition target in an engineered antimicrobial therapeutics approach for protection against *X. fastidiosa*. Nevertheless, the function of this protein remains poorly understand. ii) The *msrA* gene encodes a methionine sulfoxide reductase MsrA, an enzyme that has been found to be involved in cell adhesion by human pathogenic bacteria [27]. In *X. fastidiosa*, *msrA* gene expression of biofilm cells are downregulated approximately 3-fold in Ca supplemented media compare to that in non-supplemented media [16]. Additionally, based on genomics and X-ray microanalysis, it has been proposed that MsrA helps to maintain thiol groups on the bacterial surface, and these thiol groups interact with Ca and Mg (magnesium) to form divalent cation bridges mediating the bacterial aggregation and adhesion [24]. The putative function of MsrA in *X. fastidiosa* has not been experimentally confirmed. Overall, we hypothesized that *mopB* and *msrA* were associated with Ca regulated virulence traits of *X. fastidiosa*. Accordingly, in this study, *mopB* and *msrA* mutants in two *X. fastidiosa* strains 'Temecula' and 'WM1-1' were constructed by site directed mutagenesis. 'Temecula' is a type strain of the causal agent of Pierce's disease in grapevine; the whole genome of which has been sequenced [28]. 'WM1-1' is a more aggressive strain that was isolated by our research group from naturally infected grapevine in Georgia [29, 30]. The

response of the mutants to Ca supplementation was investigated; the effect of *mopB* and *msrA* mutations on adhesiveness, biofilm formation, twitching motility and disease development in planta were evaluated as well. Results show that *mopB* and *msrA* mutants have similar response to Ca supplementation as the WT strains. However, the *mopB* mutation impaired *X. fastidiosa* virulence traits including surface attachment, biofilm formation and twitching motility. MopB appears to be involved in pili biogenesis. Nether type I pili nor type IV pili were observed on *mopB* mutant. In addition, *mopB* deletion reduces the pathogenicity of *X. fastidiosa* under greenhouse conditions. The *msrA* mutation does not affect biofilm formation, twitching motility and pathogenicity of *X. fastidiosa.*

Materials and methods

Bacterial strains, plasmids, primers and culture conditions.

Bacterial strains and plasmids used in this study are listed in Table 3-1. All primers listed in Table 3-2 were designed by PrimerQuest software (Intergrated DNA Technologies). *X. fastidiosa* type strain 'Temecula' and a more aggressive strain 'WM1-1' [29, 30] were used as the wild-type (WT) strains. All *X. fastidiosa* strains and mutants were cultured on PW [31] or PD3 plates [32], or in PD2 broth [33] at 28°C. All cell suspensions of *X. fastidiosa* used in this study were prepared as follows: seven-day-old bacterial cultures were scraped from PW agar medium plates, suspended in PD2 broth, and diluted to OD_{600} of 1.0. All *Escherichia coli* strains were cultured on Luria-Bertani (LB, Difco^{TM}, BD company) plates or in LB broth.

Table 3-1. Strains and plasmids used in this study

Strains/plasmids	Characteristics[a]	Source
Xylella fastidiosa strain 'Temecula'	Wild-type *X. fastidiosa,*	[28]
Xylella fastidiosa strain 'WM1-1'	Wild-type *X. fastidiosa*	[29]
mopB(T)	*mopB* mutant in *X. fastidiosa* 'Temecula', knockout in PD1709, Km^R	This study
mopB(W)	*mopB* mutant in *X. fastidiosa* 'WM1-1', knockout in PD1709, Km^R	This study
msrA(T)	*msrA* mutant in *X. fastidiosa* 'Temecula', knockout in PD0859, Km^R	This study
msrA(W)	*msrA* mutant in *X. fastidiosa* 'WM1-1', knockout in PD0859, Km^R	This study
Escherichia coli NEB 5-alpha	*fhuA2 Δ(argF-lacZ)U169 phoA glnV44 Φ80 Δ(lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17*, general cloning host	New England Biolab
Escherichia coli EAM1	DH5α derivative, Sp^R St^R $attP_{HK022}$::(PLlacO-1-PD1607), expressiong the methylase (PD1607) of *X. fastidiosa*	[34]
pJET1.2	Blunt cloning vector, Amp^R	Thermo Fisher Scientific
pJET_mopB	Regions upstream and downstream of *mopB* cloned into pJET1.2 , Amp^R	This study
pJET_mopB_Kan	Kanamycin cassette cloned into Pjet_mopB, Amp^R, Km^R	This study
pJET_msrA	Regions upstream and downstream of *msrA* cloned into pJET1.2 , Amp^R	This study
pJET_msrA_Kan	Kanamycin cassette cloned into pJET_mopB, Amp^R, Km^R	This study

a: Km^R and Amp^R indicate resistance to kanamycin and ampicillin, respectively.

Table 3-2. Primers used in this study

Primer	Function/target	Sequence*	Source
Mutagenesis			
mopB-U-F	Upstream *mopB*	CGACCTTCTTTCGTATTCCTAG	This study
mopB-U-R	Upstream *mopB*	ACGGGCGCGCCATGCTTGTCACATCC AGACAG	This study
mopB-D-F	Downstream *mopB*	ACGGGCGCGCCGTAATGTGCTCCATA GCAAAGC	This study
mopB-D-R	Downstream *mopB*	ACCATTCCCTGGAAGAGTAGA	This study
msrA-U-F	Upstream *msrA*	GTACTAGTGTTGTTGGATTGGA	This study
msrA-U-R	Upstream *msrA*	ACGGGCGCGCCCGGAAACACGGGCA CAGATAC	This study
msrA-D-F	Downstream *msrA*	ACGGGCGCGCCTATCCACAGGCTCAG GTACA	This study
msrA-D-R	Downstream *msrA*	GCTAAACCACACACGCAATC	This study
KanAscIF	Kanamycin cassette	TTAGGCGCGCCGTCTGCCTCGTGAAG	This study
KanAscIR	Kanamycin cassette	AAAGGCGCGCCAAGCCACGTTGTGT	This study
Confirmation of mutations			
mopBF	*mopB* gene deletion	GTCCGTGCTGAAGTAGCTTATC	This study
mopBR	*mopB* gene deletion	CAGGGTAGCGTCTCAAGATTTC	This study
msrAF	*msrA* gene deletion	CGTTGATGAATCTCGGGTAGG	This study
msrAR	*msrA* gene deletion	GGTGCTCATTCTCAGCATAGT	This study
KanF	Kanamycin cassette replace	TCGGGAAGATGCGTGATCTG	This study
KanR	Kanamycin cassette replace	CGCGATAATGTCGGGCAATC	This study
B-U-F1	Upstream *mopB sequencing*	CCTTCTTTCGTATTCCTAGACAAC	This study
B-U-R1	Upstream *mopB sequencing*	AGACAGCAGTCCCACAC	This study
B-D-F1	Downstream *mopB sequencing*	ACGCTGTAACGCATCTTCTATAC	This study
B-D-R1	Downstream *mopB* sequencing	ATATTCTCATATGCATTGA	This study
B-UK-F	Kanamycin cassette sequencing	TGTGTGGGACTGCTGTCT	This study
B-DK-R	Kanamycin cassette sequencing	ATTCACTGTCACCTCACATCG	This study
A-U-F1	Upstream *msrA* sequencing	GGAGTACTAGTGTTGTTGGATTG	This study
A-U-R1	Upstream *msrA sequencing*	CAGAAGATTCACTTTACGGTAGTT	This study

(Continued)

Table 3-2. Continued

Primer	Function/target	Sequence*	Source
Confirmation of mutations			
A-D-F1	Downstream *msrA sequencing*	AGGAAGAGAGCAAATGGATACT	This study
A-D-R1	Downstream *msrA* sequencing	GGTAGTTAATGTATCCGTGAGGTT	This study
A-UK-F	Kanamycin cassette sequencing	CTGTTGCCAACCATGTCTAAC	This study
A-DK-R	Kanamycin cassette sequencing	TCGGTACAGGGAATGATTAAAGG	This study
Quantification of *X. fastidiosa*			
HL5	Detection of *X. fastidiosa*	AAGGCAATAAACGCGCACTA	[35]
HL6	Detection of *X. fastidiosa*	GGTTTTGCTGACTGGCAACA	[35]
HLP	Detection of *X. fastidiosa*	FAM-TGGCAGGCAGCAACGATACGGCT-BHQ	[35]

* AscI restriction site is underlined

Construction of *mopB* and ***msrA* mutants.**

Natural competence and homologous recombination were used to generate *X. fastidiosa* 'Temecula' and 'WM1-1' *mopB* (PD1709) null mutants. *mopB* mutant in 'Temecula' was named *mopB*(T) and in 'WM1-1' was named *mopB*(W). For *msrA* (PD0859) null mutants, *msrA* mutant in 'Temecula' was named *msrA*(T) and in 'WM1-1' was named *msrA*(W).

(i) *mopB* and *msrA* knockout vectors. Genomic DNA of WT strain 'Temecula' was extracted using a modified cetyltrimethylammonium bromide (CTAB) protocol [36]. 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 ligated with T4 ligase (Thermo Fisher Scientific, Pittsburgh, PA). The ligated product was cloned into pJET1.2/blunt cloning vector (Thermo Fisher Scientific, Pittsburgh, PA). The resulting plasmid, pJET_mopB was transformed into *E. coli* NEB 5-alpha (New England Biolabs, Ipswich, MA) and positive clones were selected in 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 dephosphorylated with shrimp alkaline phosphatase (Affymetrix, USB, Santa Clara, CA). The kanamycin segment was also digested with AscI, and ligated to the digested pJET_*mopB* by T4 ligase. The final plasmid pJET_mopB_Kan was transformed into *E. coli* NEB 5-alpha and positive clones were selected in LB medium plate with kanamycin (50 µg/ml).

The *msrA* knock out vector, pJET_msrA_Kan, was constructed as described above using primers msrA-U-F /msrA-U-R and msrA-D-F/msrA-D-R for amplification of the upstream and downstream segments flanking the *msrA* open reading frame and addition of AscI restriction site.

(ii) Natural competence. *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 [37]. Briefly, pJET_mopB_Kan was transformed in *E. coli* EAM1 to allow *in vivo* methylation of the plasmid [34]. 10 µl cell suspension (OD_{600} = 1.0) of WT strain was mixed with 5 µg modified pJET_mopB_Kan that isolated from *E. coli* EAM1, and spotted on 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 suspended in 250 µl water, and serial dilutions were made. 150

µl of each dilution were plated on PD3 medium plate with kanamycin (50 µg/ml). Plates were incubated at 28°C until transformants were visible (~10 days). *msrA* knockout vector pJET_msrA_Kan was transformed into *X. fastidiosa* 'Temecula' and 'WM1-1' by the same method.

(iii) Confirmation of *mopB* and *msrA* null mutants. DNA was extracted from the resulting transformants in 'Temecula' and 'WM1-1' backgrounds using the CTAB protocol mentioned above. Primers mopBF/mopBR (msrAF/msrAR) and KanF/KanR designed from the *mopB* (*msrA*) coding region and kanamycin cassette were used to confirm the deletion of *mopB* (*msrA*) gene. Primers mopB-U-F1/mopB-U-R1, mopB-D-F1/ mopB-D-R1 and B-UK-F/B-DK-R or msrA-U-F1/ msrA -U-R1, msrA -D-F1/ msrA -D-R1 and A-UK-F/A-DK-R were used to sequence the homologous recombination region (Eurofins MWG Operon, Huntsville, AL). 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.

Measurement of cells growth

For each strain of *X. fastidiosa*, 0.1 ml cell suspension (OD_{600} = 1.0) was added to 50 ml conical tubes filled with 9 ml PD2 broth. Each strain was inoculated in three tubes. All tubes were incubated at 28°C with shaking at 150 rpm. The OD_{600} was measured at 1, 3, 5, 7, 9 days after incubation. Two independent experiments were performed.

Biofilm formation in glass tube and 96-well plates

To compare the phenotype of biofilm formed by *X. fastidiosa* WT strains and mutants, 0.1 ml cell suspension (OD_{600} = 1.0) was added in glass tube filled with 9 ml PD2 broth. Inoculated tubes were incubated at 28°C and shaken with a speed of 150 rpm for 5 days. Biofilm formed at the liquid-air interface was recorded. Two independent experiments were performed.

Biofilm formation in 96-well plates was quantified according to the method previously described with some modifications [10]. 10 µl cell suspension (OD_{600} = 1.0) was inoculated in one well of a sterile 96 well plate. Each well contained 190 µl of either media: PD2 broth (PD2), PD2 broth supplemented with 4 mM $CaCl_2$ (4Ca), *Vitis vinifera* 'Chardonnay' grape xylem fluid mixed with equal volume of PD2 broth (50% Sap), and full strength Chardonnay grape xylem fluid (Sap). For each medium, each strain was inoculated in 12 wells considered as repetitions. 96 well plates were incubated in 28°C and shaken with a speed of 150 rpm for 7 days. The planktonic phase was transferred to a new plate. The original plate was rinsed three times with sterile Milli-Q water. Biofilm was stained with 250 µl 0.1% crystal violet for 20 min, and rinsed with distilled water as described above. 210 µl of 6:4 (vol/vol) acetone/ethanol were added, and the plates were agitated at 150 rpm for 5 min. The OD_{600} of the resulting acetone/ethanol solution was measured. There independent experiments were performed.

Bacterial cell-cell aggregation analysis

The cell-cell aggregation assay was performed as previously described [38]. 200 µl of cell suspension (OD_{600} = 1.0) was added into a 50 ml conical tube filled with 10 ml PD2 broth or PD2 broth supplemented with 4mM $CaCl_2$. Tubes were incubated at 28°C without shaking. Five

days after incubation, all tubes were vortexed and the OD_{600} (OD_t) of each tube was measured. After these tubes were standing for 20 min to allow the aggregated cells to settle, the OD_{600} (OD_S) of supernatant of each tube was measured again. The relative percentage of aggregated cells was calculated using the formula: Cell aggregation rate = $[(OD_t - OD_S) \times 100] / OD_t$ [39]. 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 which reflects the strength of cell attachment to surfaces. This parameter was evaluated using microfluidic chambers as previously described [10, 40]. The microfluidic chamber consists of a molded polydimethylsiloxane (PDMS) body and a glass microscope slide. There are two parallel microchannels (80 μm wide x 3.7 cm long x 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 strain 'Temecula' and *mopB*(T) were suspended in PD2 broth. PD2 broth was filled into a 5 ml glass syringes. 'Temecula' cell suspension was filled into a 1 ml plastic syringe. PD2 broth and 'Temecula' cell suspension were introduced into one microchannel by two automatic syringe pump (Pico Plus; Harvard Apparatus, Holliston, MA). Accordingly, PD2 broth and the *mopB*(T) cell suspension were introduced into another microchannel. 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, Melville, NY) and observed under a 40X objective using Nomarski differential interference contrast (DIC) optics and phase contrast. After the bacterial cells entered the channels, media flow speed was maintained at 0.25 μl min^{-1} for approximate 2 hours. When enough cells attached to the slide surface, media speed was adjusted to 1.0 μl min^{-1} for 1 hour to remove non-attached cells. Media flow was then sequentially increased every 1 min by 10 μl/min from 1-160 μl/min to gradually remove attached cells. During this process, time-lapse microscopy images were acquired every 5 seconds using a Nikon DS-Q1 digital camera (Nikon, Melville, NY) controlled by NIS-Elements Advanced Research 3.01(Nikon, Melville, NY). The number of attached cells remaining in each frame was counted. The number of attached cells for each flow rate was equal to the average cell numbers of 12 frames collected in 1 minute. Cell adhesion force was calculated according previous method described by De La Fuente et al. [40]. Three independent microfluidic chamber experiments were performed.

Quantification of twitching motility

Twitching motility of *X. fastidiosa* strains was evaluated on PW medium plate without bovine serum albumin (BSA) as previously described [10]. For each strain, bacterial cells were spotted in quadruplicate on triplicate plates. After incubating at 28°C for two days, peripheral fringe of each spot was observed under a 40X phase-contrast optics on Nikon Eclipse Ti inverted microscope (Nikon, Melville, NY). 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 [10, 40]. Cell suspension of 'Temecula' and *mopB*(T) were separately introduced into different channel of the chamber. Media flow rate was controlled at 0.25 μl min^{-1} for 2 hours to allow cells attached on the surface. Time-lapse images were then acquired every 30 seconds for 2 or 3 hours by Nikon DS-Q1 digital camera. Using NIS-Elements Advanced Research 3.01, the upstream movement of bacterial cells can be quantified according their position frame by frame. The twitching speed was calculated through dividing the upstream movement by time. Three independent experiments were performed.

Transmission electron microscopy

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

Greenhouse experiment

All *X. fastidiosa* strains were inoculated to tobacco plants (*Nicotiana tabacum* 'Petite Havana SR1', plant introduction number 552516) to evaluate their virulence according to a protocol previously described [41]. Seeds were provided by United States Department of Agriculture (USDA) Germplasm Resources Information Network. After seeds were geminated in SunshineH Mix #8 (Sun Gro Horticulture Canada Ltd., Vancouver, Canada), seedlings were transplanted in

4.5 inch 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, Marysville, OH, U.S.A.) was applied one time at 40 to 45 days post-transplant. When tobacco plants have five to six true leaves, the top of stem and leaves were removed. The lowest three of the true leaves were kept and inoculated with *X. fastidiosa* cells suspended in 1.5 ml succinate-citrate phosphate buffer (OD_{600} of 1.0) by 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 greenhouse for two or three month until leaf scorch symptom appeared. Plants symptoms were then assessed weekly for four weeks. Disease incidence was represented by the percentage of infected plants in the eight plants inoculated with the same strain. Symptoms of the eight leaves above the site where the stem was cut were recorded, including leaf scorch, leaf curling or cupping, and intraveinal chlorosis. A score was given to each assessed leaf according the following standard: leaf scorch area more than 50% was two points, leaf scorch area less than 50 % was one point, added one point if obvious leaf curling or cupping was observed, and added one point if obvious interveinal chlorosis was observed. Disease severity score of each plant equal to average score of the eight assessed leaves. An average disease severity (ADS) score of infected plants by the same strain was calculated. According to ADS score at each time point, the value of area under the disease progress curve (AUDPC) was calculated for each strain.

To determine if inoculated plants became infected with *X. fastidiosa*, a quantitative polymerase chain reaction (qPCR) analysis was performed as described previously [30, 41]. Total DNA was extracted from petioles of four leaves from above the inoculation point, using a modified CTAB protocol [36]. Leaves were collected at the end of the fourth week of symptom monitoring and sections of the lower petioles were excised. For each strain, three infected plants were used for leaf collection. Four leaves were collected from each plants, and *X. fastidiosa* CFU per gram of extracted petiole tissue were quantified using qPCR and a standard curve as previously described [41].

In addition, *X. fastidiosa* was isolated from infected plants using a method described previously [29]. 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 and shaking with 150rpm for 1 hour. The supernatant was diluted and spread-plated on agar PW medium and PW with kanamycin (50 $\mu g\ ml^{-1}$).

Statistical analysis

Data from biofilm production in 96-well plates, fringe width, and cell aggregation were analyzed by SAS 9.4 (SAS Institute Inc., Cary, NC, U.S.A.) 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 AUDPC between mutant and corresponding WT strain, data were analyzed by *t* test ($P < 0.05$).

Results

Generation of *mopB*$^{-}$ and *msrA*$^{-}$ mutants of *X. fastidiosa* 'Temecula' and 'WM1-1'

Taking advantage of natural competence of *X. fastidiosa*, the gene knockout vectors pJET_mopB_Kan or pJET_msrA_Kan were directly introduced to *X. fastidiosa* 'Temecula' and 'WM1-1'. Two transformants per WT/gene mutated combination were selected and purified by repeated streaking on PD3 medium agar plates with kanamycin (50 µg/ml). One transformant from each background was selected to evaluate homologous recombination at the *mopB*/*msrA* locus by PCR analysis. DNA of WT strain and *mopB* mutants were amplified using Primer mopBF/mopBR (Fig 3-1A), specific bands can be detected from WT strains but not from *mopB* mutants. DNA of WT strains and *msrA* mutants were amplified using Primer msrAF/msrAR (Fig 3-1B), specific bands were only detected from WT strains. DNA of WT strains, *mopB* mutants, and *msrA* mutants were amplified using Primer KanF/KanR (Fig 3-1C), specific bands were detected from all mutants. The result shown that *mopB* or *msrA* genes were missing in the mutants, and a kanamycin cassette was introduced into them. In addition, the homologous recombination region has been sequenced, confirming that the kanamycin cassette interrupted the *mopB* /*msrA gene* in mutants (data not shown).

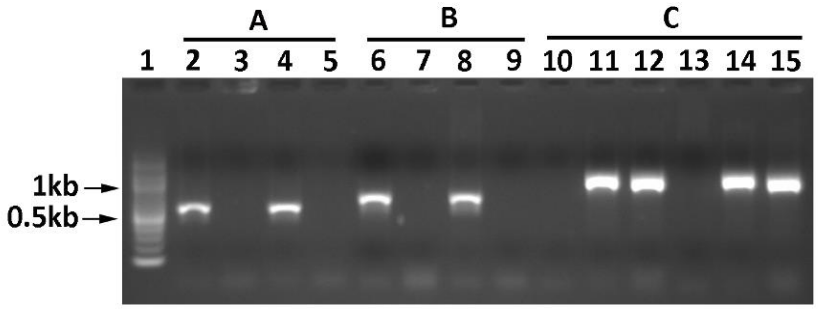

Figure 3-1. Detection of *mopB* and *msrA* gene deletion using PCR analysis. PCR using primers mopBF/mopBR, msrAF/msrAR and KanF/KanR were labeled A, B, and C, respectively.

Lane 1: 100bp DNA Ladder; Lane 2, 6, 10: *X. fastidiosa* 'Temecula'; Lane 4, 8, 13: *X. fastidiosa* 'WM1-1'; Lane 3, 11: *X. fastidiosa mopB*(T) mutant; Lane 5, 14: *X. fastidiosa mopB*(W) mutant; Lane 7, 12: *X. fastidiosa msrA*(T) mutant; Lane 9, 15: *X. fastidiosa msrA*(W) mutant.

After confirming the mutations, growth of WT strains and mutants was evaluated. The results shown that *mopB* mutants grew at the same rate as the WT strains until ~3 days post inoculation (dpi). OD_{600} of *mopB* mutants increased slower than that of WT strains after 3 dpi (Fig 3-2A). Over the nine days assay period, a thin layer of biofilm was formed by the WT strains at air-liquid interface at 3 dpi, then the biofilm become denser at 5 dpi and gradually detached from the wall at 7 dpi. However, biofilm formed by *mopB* mutants was rarely observed at the air-liquid interface throughout the period. *msrA* mutants grew at the same rate similar to the WT strain throughout the whole assay (Fig 3-2B).

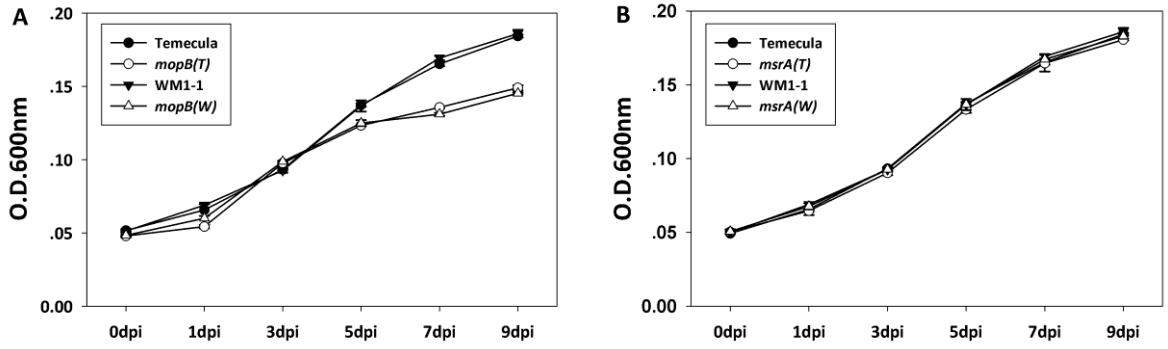

Figure 3-2. Growth of *X. fastidiosa* WT strains, *mopB* mutants (A) and *msrA* mutants (B) in 50 ml conical tubes filling with 10 ml PD2 broth for 9 days. dpi: days post inoculation. Each data point represents means (n = 9) of OD_{600} of the total bacterial culture (biofilm and planktonic phase) of each strain from one representative experiment; error bars correspond to standard error (SE) of the mean.

Effect of *mopB* or *msrA* mutation on biofilm formation of *X. fastidiosa*

For *X. fastidiosa* strains cultured in PD2 broth (PD2), biofilm growth at the air-liquid interface of the glass tube by WT strains and *msrA* mutants was obviously visible (Fig 3-3 A). Rarely biofilm was formed at the air-liquid interface by both *mopB* mutants (Fig 3-3 A). In 4mM Ca supplemented PD2 broth (4Ca), the biofilm formed by all *X. fastidiosa* strains was thicker than that in PD2 (Fig 3-3 A).

In 96 well plates, biofilm growth of each strain was quantified. In all of 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 that of the corresponding WT strains (Fig 3-3 B). In the three media containing more Ca than PD2, biofilm growth of each strain was significantly increased ($P < 0.05$). The same phenotypes were observed when strains were grown in glass test tubes. No significant difference ($P > 0.05$) of the biofilm formation was observed in WT strains and *msrA* mutants (Fig 3-3 C). Similar to WT strains, Ca supplementation increased the biofilm growth in *msrA* mutants.

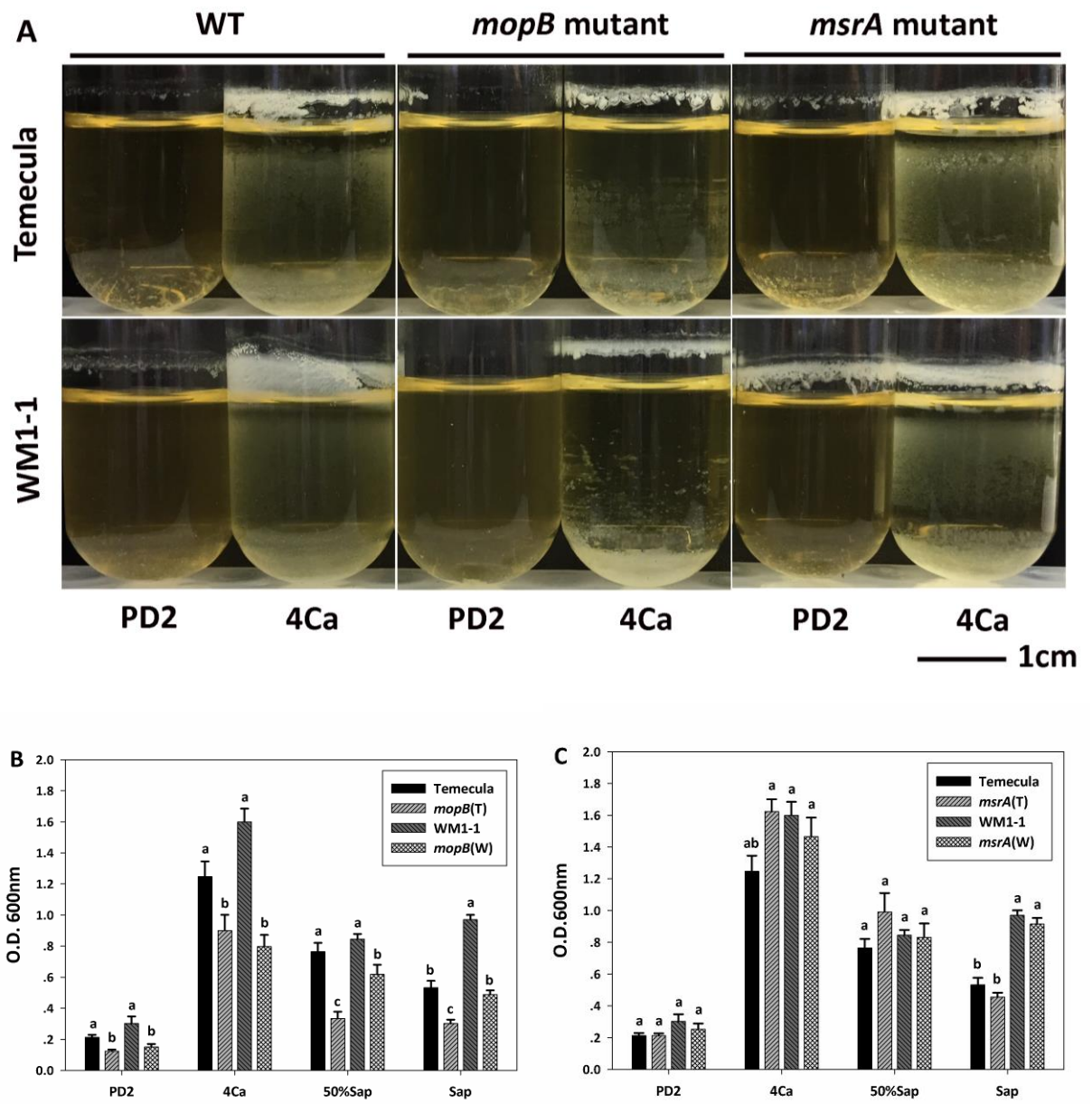

Figure 3-3. Biofilm formation of *X. fastidiosa* strains in glass tube and 96-well plate filling with different Ca concentration media. Representative picture of biofilm (**A**) forming by each strain at air-liquid interface of glass tube at 5 days after inoculation. Biofilm quantification of WT strains, *mopB* mutants (**B**), and *msrA* mutants (**C**) cultured in 96-well plate for 7 days. PD2, 4Ca, 50%Sap and Sap represent PD2 broth, PD2 broth supplemented with 4mM CaCl2, 50% 'Chardonnay' grape xylem fluid and 100% 'Chardonnay' grape xylem fluid. Each data point represents means (n = 12) from one representative experiment; error bars correspond to standard

error (SE) of the mean. Different letters indicate significant differences ($P < 0.05$) of the biofilm formation between *X. fastidiosa* strains. Analysis was performed by the GLIMMIX procedure using SAS 9.4 (SAS Institute Inc).

MopB is important for cell aggregation and surface attachment.

Surface attachment and cell aggregation are initial stages in the process of biofilm formation [42]. The strength of surface attachment was assessed by measuring the percentage of attached cells and adhesion force for 'Temecula' and *mopB* (T) using microfluidic chambers. msrA mutants were not assessed in these characteristics because they did not show an impediment in biofilm formation. When the flow rate reached 40 µl min^{-1}, the percentage of attached *mopB* (T) cells decreased dramatically (Fig 3-4A). In contrast, with the increase of flow rate, the percentage of attached 'Temecula' cells dropped slowly (Fig 3-4A). When the adhesion force (AF) was calculated, a significant difference ($P = 0.02$) was observed between 'Temecula' (221.8 pN) and *mopB* (T) (139.4 pN) (Figure 3-4B).

Cell aggregation of Temecula and *mopB* (T) were observed in microfluidic chambers (Figure 3-4C) and quantified in 50 ml conical tube (Fig 3-4D). At 5 days post inoculation, aggregates of 'Temecula' cells were observed in several sections of the microchannel. In contract, aggregates of *mopB* (T) cells were rarely observed and the size of the aggregate was much smaller than that of 'Temecula'. Cell aggregation of the WT and mutant strains was quantified by calculating the relative percentage of aggregated cells in conical tube (Fig 3-4D). The percentage of aggregated cells of *mopB* mutants was significantly lower ($P < 0.001$) than that of the WT stains in PD2. However, in 4Ca, there is no significant deference between the WT and mutant.

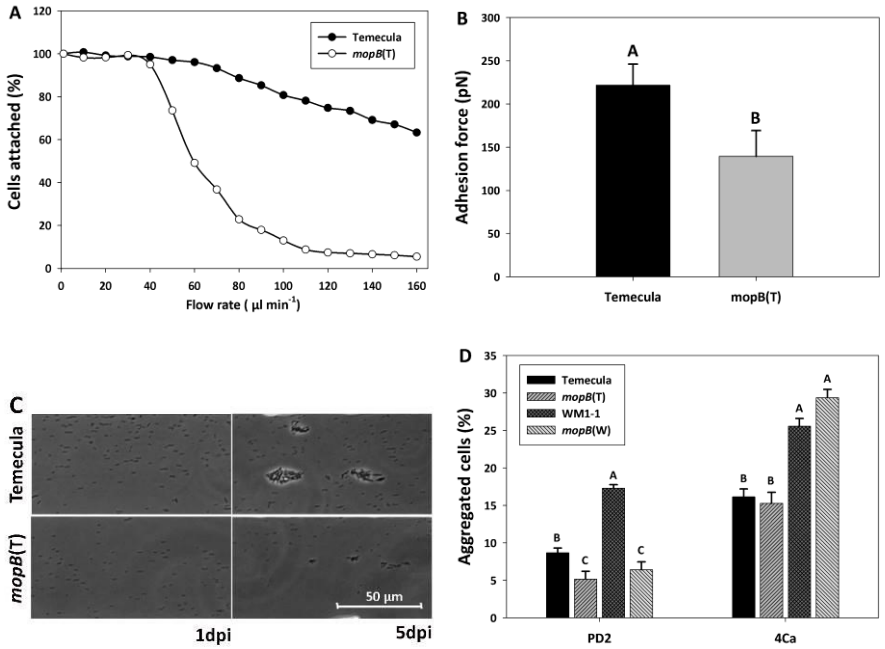

Figure 3-4. Evaluation of cell aggregation and surface attachment of *X. fastidiosa* WT and *mopB* mutant. A) Change of attached cells on the surface of microfluidic chamber as a function of increased medium flow rate. The data represented in the graph is from one representative experiment. Three independent experiments have the same tendency. **B**) Adhesion force of *X. fastidiosa* Temecula and *mopB* (T) assessed in microfluidic chambers. Different letters on the bars indicate significant differences ($P < 0.05$) according to Student's *t*-test. Error bars represent standard errors of the mean (n = 3) **C**) Time lapse micrographs showing formation of cells aggregate inside microfluidic chambers. Images were captured at 1 and 5 days after inoculation (dpi). **D**) The percentage of aggregated cells of WT strains and *mopB* mutants cultured by PD2 and 4Ca medium in 50 ml conical tube. Each bar represents means (n = 3) from one representative experiment; error bars correspond to standard error (SE) of the mean. Different letters on the bars indicate significant differences ($P < 0.05$) according to the GLIMMIX procedure using SAS 9.4

mopB mutants become non-motile

The colony fringe width on agar plates is a parameter indicating the twitching motility of *X. fastidiosa*. From the image of the colonies, peripheral fringe was observed for WT 'Temecula' and 'WM1-1'. But the edge of *mopB* mutant colonies was smooth and there was no visible peripheral fringe (Fig 3-5A). The width of the fringe in 'WM1-1' (mean = 180 μm) was twice as much as that of 'Temecula' (mean = 90 μm) fringe size (Fig 3-5B).

In microfluidic chambers, the twitching movement of single cells was visible. 'Temecula' cells in the channel of microfluidic chamber not only move following medium flow, but also move against the flow direction. In contrast, the upstream movement of *mopB* (T) cells was not observed (Support video 1). The average twitching speed of Temecula cells was 0.45 μm min^{-1} (Fig 3-5C). The results obtained from microfluidic chamber experiment correlates with the colony fringe observations.

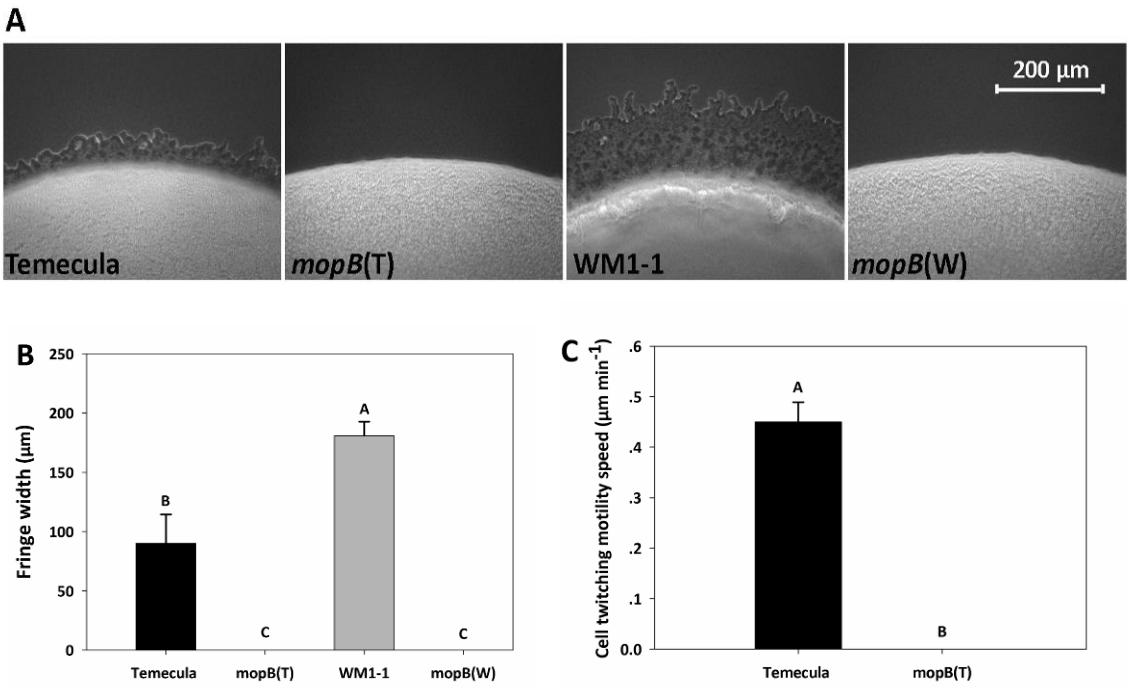

Figure 3-5. Twitching motility of *X. fastidiosa* WT strains and *mopB* mutants. A) Representative micrographs of colony fringe of on PW without BSA plates. **B**) Colony fringe width of *X. fastidiosa* WT strains and *mopB* mutants on PW without BSA plates. Each bar represents means (n = 36) from one representative experiment; error bars correspond to standard error (SE) of the mean. Different letters on the bars indicate significant differences ($P < 0.05$) according to the GLIMMIX procedure. **C**) Twitching speed assessments of *X. fastidiosa* Temecula and *mopB* (T) in microfluidic chambers. Different letters on the bars indicate significant differences ($P < 0.05$) according to Student's *t*-test. Error bars correspond to standard error (SE) of the mean (n = 9).

The *mopB* mutation affect pilus biogenesis of *X. fastidiosa*

Transmission electron micrographs revealed the presence of two types of pili on one polar end of *X. fastidiosa* WT strains. They are short type I pili and long type IV pili. In contrast, no pilus was observed on the polar end of *X. fastidiosa mopB* mutants (Fig 3-6).

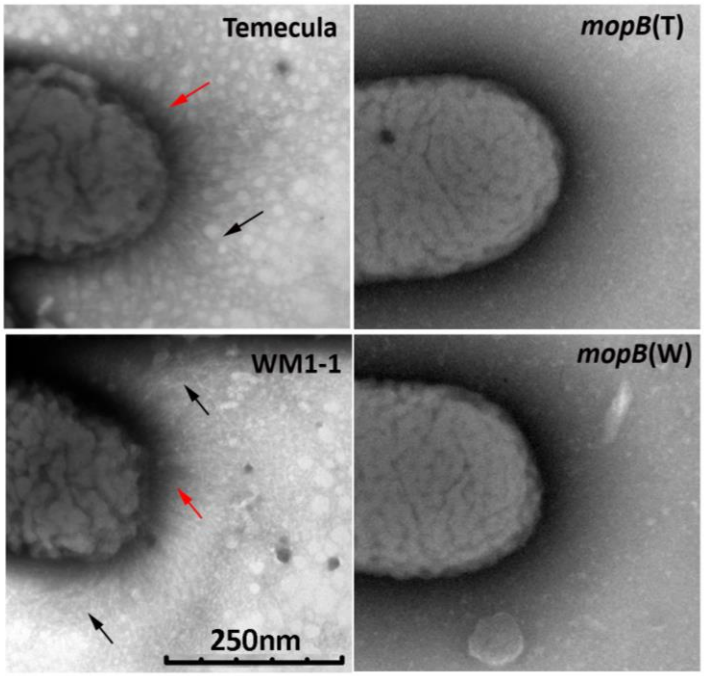

Figure 3-6. Representative Transmission electron micrograph of negatively stained cells of *X. fastidiosa* WT strains and *mopB* mutants. Red arrow point type I pili and Black arrow point type IV pili. The magnification is 40000×.

msrA mutation has no effect on the twitching motility by *X. fastidiosa*

Similar peripheral morphology was observed in WT strains and the *msrA* mutants from assessing colony fringe (Fig 3-7A). Quantification of the colony fringe width indicates that there was no significant difference ($P > 0.05$) between the WT strains and *msrA* mutants (Fig 3-7B).

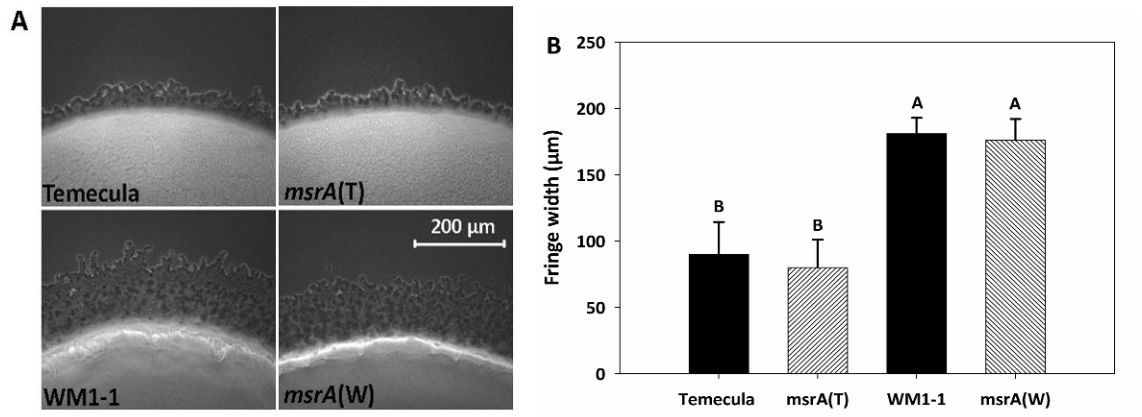

Figure 3-7. Twitching motility of *X. fastidiosa* WT strains and *msrA* mutants. A) Representative micrographs of colony fringe of on PW without BSA plates. **B**) Colony fringe width of *X. fastidiosa* WT strains and *msrA* mutants on PW without BSA plates. Each bar represents means (n = 36) from one representative experiment; error bars correspond to standard error (SE) of the mean. Different letters on the bars indicate significant differences ($P < 0.05$) according to the GLIMMIX procedure.

mopB mutation causes virulence decrease of *X. fastidiosa*

Previous reports indicate tobacco as a model plant suitable for estimating virulence of *X. fastidiosa* strains and investigating *X. fastidiosa*-host interactions [41, 43, 44]. 'Petite Havana SR1' tobacco plants inoculated with the *X. fastidiosa* strains showed symptoms including leaf scorch, leaf curling and intraveinal chlorosis. The earliest symptoms were observed on the WT inoculated plants approximately 70-75 days after inoculation. One or two weeks later, symptoms were visible on the *mopB* (W) and *mopB* (T) inoculated plants (Fig 3-8A). 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 3-8A). ADS score indicates disease severity of infected plants. According to ADS score, disease progress curve for

each WT strains and *mopB* mutants was made. AUDPC value of WT strains were significantly higher ($P < 0.001$) than that of *mopB* mutants (Fig 3-8B).

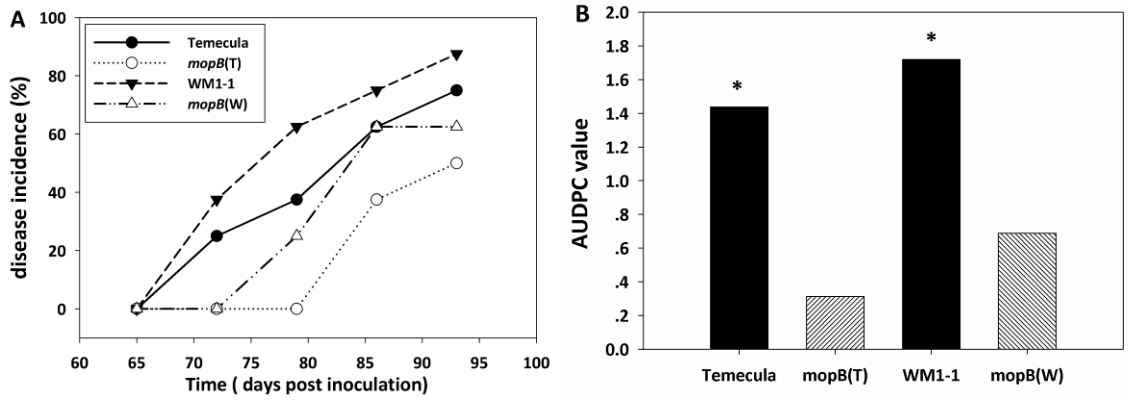

Figure 3-8. The virulence study of *X. fastidiosa* WT strains and *mopB* mutants on tobacco plant in greenhouse. A) Disease incidence of tobacco plant infected by *X. fastidiosa* each strains in continued 4 weeks after first leaf scorch symptom were evident. Disease incidence is represented by the percentage of plant with symptoms. The data represented in the graph was obtained from one representative experiment, two independent experiments have the same tendency. **B**) Calculated AUDPC of disease on tobacco plants caused by infection of *X. fastidiosa* WT strains and *mopB* mutants. Asterisk indicates a significant difference ($P < 0.05$) between each pair WT strain and *mopB* mutant. Analysis was performed using Student's *t*-test.

Virulence test of *msrA* mutants

According to disease incidence and calculated AUDPC, there was no significant difference in the disease severity and disease progress between the plants infected by *X. fastidiosa* WT strains and *msrA* mutants (Fig 3-9).

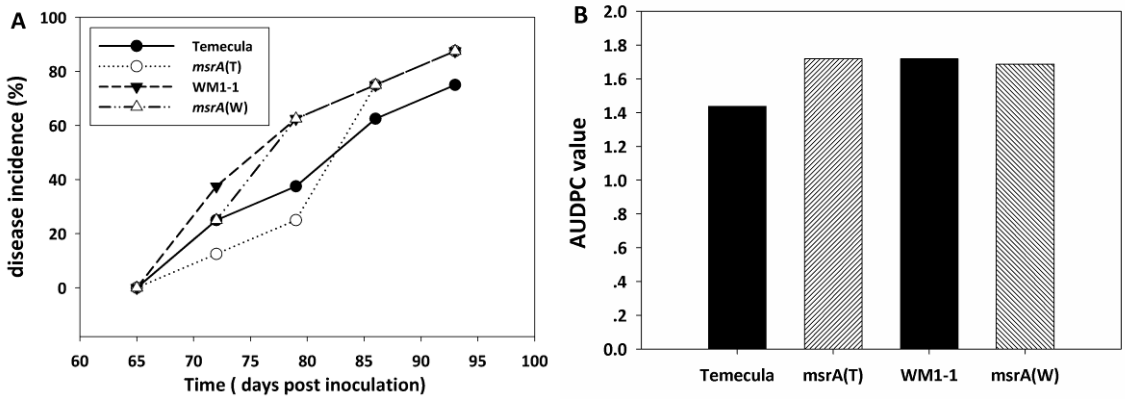

Figure 3-9. The virulence study of *X. fastidiosa* WT strains and *msrA* mutants on tobacco plant in greenhouse. A) Disease incidence of tobacco plant infected by *X. fastidiosa* each strains in continued 4 weeks after first leaf scorch symptom shown. Disease incidence represented by the percentage of plant with symptoms. The data obtained from one representative experiment, two independent experiments have the same tendency. **B**) Calculated AUDPC of disease on tobacco plants caused by infection of *X. fastidiosa* WT strains and *msrA* mutants.

Colonization of *X. fastidiosa* in tobacco plants

qPCR detection indicated 100% of the infected plants were colonized by *X. fastidiosa.* There were no significant difference of the bacterial population in infected plants by WT strains, *mopB* mutants, and *msrA* mutants. For plant infected with 'Temecula', *mopB*(T) and *msrA*(T), the average bacterial population detected in infected plants leaf petioles are 7.2±0.06, 7.0±0.07 and 7.4±0.05 log CFU g^{-1}, respectively (Fig 3-10). The average bacterial population detected in infected plants leaf petioles was 6.8±0.02, 6.9±0.06 and 6.9±0.06 log CFU g^{-1} for plant infected with 'WM1-1', *mopB*(W) and *msrA*(W), respectively (Fig 3-10). In addition, *X. fastidiosa* isolated from plants infected by WT strains grew on PW plate, but did not grow on PW plate with 50 μg ml^{-1} kanamycin. This indicated that these isolates did not have kanamycin resistance. *X. fastidiosa* isolated from plants infected by mutants not only grow on PW plates but also grow

on PW plates with 50 µg ml^{-1} kanamycin, confirming that these isolates have kanamycin resistance.

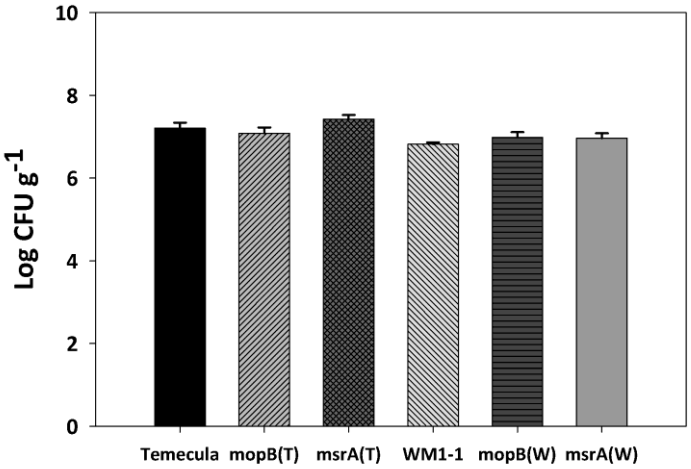

Figure 3-10. Bacterial colonization of tobacco. Tobacco plants were inoculated with *X. fastidiosa* WT strains, *mopB* mutants, and *msrA* mutants and maintained in the greenhouse for approximately 90 days. Bacterial populations were quantified from leaf petioles of infected plants by qPCR. Each bar represents means (n = 12) from one representative experiment; error bars correspond to standard error (SE) of the mean.

Discussion

In this study, we assessed the role of two genes *mopB* and *msrA* in virulence traits and disease development of two *X. fastidiosa* stains, 'Temecula' and 'WM1-1'. *mopB* deletion in either *X. fastidiosa* strain impaired growth, biofilm development, twitching motility, pilus biogenesis and virulence. *msrA* deletion in both strains had no significant effect on the virulence traits and disease development. A priori, based on the characteristics of these two genes and previous studies [16, 24], *mopB* and *msrA* were proposed to play a role in the Ca regulation of biofilm,

movement and virulence. However, our results indicate that these genes are not responsible for the Ca-mediated response of these characteristics, although *mopB* was proven here to be fundamental for biofilm, movement and virulence, regardless of the Ca concentration in the media.

mopB encodes a major outer membrane protein MopB which is the most abundant protein in *X. fastidiosa* [26]. Outer membrane, a specific structure locates at the outside of gram negative bacteria, is an asymmetric lipid bilayer constructed by phospholipids in its inner leaflet and lipopolysaccharide (LPS) in its outer leaflet [45]. The membrane serves as a protective barrier and controls the translocation of materials as well as signal transduction, which is important for the survival of gram negative bacteria in their natural environments [46]. Outer membrane proteins (OMPs) serve as channels vital for the intake of nutrients and the excretion of toxic wastes and are one of two types proteins in the outer membrane. Besides the function of channels, OMPs also serve as enzymes, adhesins, and maintenance of membrane integrity [46, 47]. Deficiency of MopB may affect outer membrane integrity of bacteria. Chen et al. [25] reported that *mopB* mutation caused a less compact outer membrane, leading to an enlarged periplasmic space in *Xanthomonas campestris* pv. *campestris*, which could be the reason of that the *mopB* mutant of *X. campestris* pv. *campestris* increased sensitivity to stressful conditions. Belaaouaj et al. [48] reported one mechanism of neutrophil elastase mediated killing of *Escherichia coli* that is degradation of outer membrane protein A (OmpA), which is a homologue of the C-terminal part of MopB, leading a loss of membrane integrity. In the present study, *mopB* deletion reduce the growth of *X. fastidiosa* and appears to be associated with

changes of outer membrane integrity; however, further studies are needed to confirm the effect of *mopB* mutation on outer membrane integrity of *X. fastidiosa*.

Biofilm is essential for *X. fastidiosa* inhabiting in xylem environment and leading to disease development. Biofilm formation of this pathogen has been extensively studied, and a number of studies indicated that surface attachment and cell aggregation are important in the early stages of biofilm development [10, 38]. Experiments from the current study shown that *mopB* deletion decreases the strength of the bacterial attachment to surface, the percentage of cell to cell aggregation, as well as biofilm formation. These effects of *mopB* deletion could be related with the changes of cell surface structure, such as fimbriae and afimbrial adhesins, which play an important role in biofilm formation [49]. Former studies reported that *X. fastidiosa fimA* null mutant, without type I pilus, significantly reduced the strength of surface attachment [40]. In this study, type I and type IV pili were not observed on the cell polar end of *mopB* mutants using transmission electron microscopy. Even though type IV pili mainly contribute to cell motility, they also play a role in cell adhesion and biofilm formation [50, 51]. In addition, some proteins located in outer membrane that act as afimbrial adhesins, such as hemagglutinins, have been shown to be crucial for cell aggregation and biofilm formation [52]. We hypothesize that MopB may be a kind of afimbrial adhesin mediating cell aggregation of *X. fastidiosa*; however, directly experimental evidences are needed to provide for this hypothesis.

Twitching motility is another important factor for survival and virulence of *X. fastidiosa*, which occurs by the extension, tethering and then retraction of polar type IV pili [53]. The speed of the cell movements and colony fringe width demonstrated that *mopB* deletion caused a loss of

twitching motility. These results were confirmed by the pili observation using transmission electron microscopy; no pili were observed on the pole of *mopB* mutant cells. Pili are complex hair-like appendages located on the surface of many bacteria. The biogenesis of type IV pili has been extensively studied in several bacteria, such as *Pseudomonas aeruginosa* and *Myxococcus xanthus*. At least 12 proteins involved in assembly of a single type IV pilus; and a number of other proteins related with the function and regulation of type IV pili [51, 54]. PilA, the basic structural unit composed type IV pilus filament, is secreted to the cell surface through the outer membrane secretin. The protein complex consisted of OMP PilQ subunits and its lipoprotein pilotin PilF [51, 54]. MopB is the most abundant protein in outer membrane. We proposed that deficiency of MopB may be influence the structure and/or function of the outer membrane secretin. Therefore, pilus biogenesis was affected. However, this proposal is needed to confirm by experimental evidence.

The results of in vitro experiments suggested that MopB is important for the virulence traits of *X. fastidiosa*. Correspondingly, in *in vivo* experiments, *mopB* mutation reduces the virulence of both *X. fastidiosa* strains. Compared to tobacco plants infected by WT strain, the speed of disease development and the disease severity of tobacco plants infected by *mopB* mutants were slower and milder. Similarly, *mopB* deletion in *X. campestris* pv. *campestris* also caused defects in its pathogenesis [25]. However, according to the results of qPCR determination, *mopB* deletion had no effect on *X. fastidiosa* population in the leaf petiole, showing that the mutants can still colonize the plants.

As previously described, the sequence pattern Dx[DN]xDxxxxxx[DE], representing a EF-hand motif, was found in C-terminal of MopB. *Kingella kingae* PilC2 protein, containing the same sequence pattern, was a Ca binding protein; mutation of the amino acid residue in the sequence pattern eliminated Ca binding effect of the protein and influenced twitching motility of the pathogen [22]. In this study, the effect of *mopB* mutation on biofilm formation of *X. fastidiosa* was independent of Ca. In PD2 broth, biofilm formation of *mopB* mutant was less than that of WT strain. In the other three medium tested [4Ca, 50% Chardonnay xylem fluid (50% Sap), and Chardonnay xylem fluid (Sap)], Ca concentrations are approximately 1.5-4 mM, which are much higher than PD2 broth (~0.02 mM) [11]. With the increase of Ca, the biofilm formed by WT strains and *mopB* mutants were increased. However, the similar difference of the biofilm growth between *mopB* mutants and the corresponding WT strains were still present. The results suggest that MopB does not involve in Ca-increased biofilm formation of *X. fastidiosa*. In contrast, *mopB* deletion reduced cell aggregation was recovered by growth in high Ca concentration. In PD2 broth, the percentage of aggregated *mopB* mutant cell was less than that of WT strain; but in 4mM Ca condition, the difference was eliminated. This may indicate that Ca is acting as a "glue" at the surface, but without interactions with MopB. Ca is a divalent cation, that can work as ion bridge between bacterial cells, which contributes to the bacterial adhesion [24]. Recently, it has been reported that Ca binding to extracellular DNA mediates bacterial aggregation in a variety of bacterial strains [55].

msrA encodes peptide methionine sulfoxide reductase MsrA, which is an enzyme repairing oxidative damage of protein [56]. Enzyme MsrA has been reported to be required for full virulence of some pathogenic bacteria; since the enzyme can protect the pathogen against the

host defense reaction, in particular against the production of reactive oxygen species [56-58]. MsrA mutation in *Streptococcus pneumoniae*, *Neisseria gonorrhoeae*, *Escherichia coli* and *Erwinia chrysanthemi* strains were defective in adhesion to the host cell [27, 56], which indicated that MsrA also play a role in bacterial adherence. Additionally, *msrA* mutant in *E. chrysanthemi* reduces the motility of the plant pathogen on solid surface [56]. However, in this study, *msrA* deletion had no effect on biofilm formation, twitching motility and virulence of *X. fastidiosa*. Similarly, *msrA* mutation in an oral pathogen *Actinobacillus actinomycetemcomitans* does not affect phenotypes of this pathogen including resistance to oxidative stress and adhesion to epithelial cells [59]. The results suggest that *msrA* in *X. fastidiosa* may be a pseudogene or other genes have similar function to *msrA*. Analysis of the DNA sequence of *msrA* in *X. fastidiosa* shows that the gene has a complete open read frame. Previous studies reported that some factors, including passages in axenic culture, cultural condition and Ca concentration, can regulate the expression of *msrA* gene at transcription level [16, 60, 61]. The transcript of *msrA* was observed in the whole transcriptome analysis conducting by our research group, which indicate that this gene is transcribed [16]. In addition, through analyzing alignment of MsrA sequence in *E. coli*, *E. chrysanthemi*, *X. fastidiosa* '9a5c', *X. fastidiosa* 'Temecula' and 'WM1-1', the active site GCFWG and several specific cysteine residues [62, 63], which could play important roles in the enzyme activity, were conserved in all of these strains (Fig 3-11). Actually, in many organisms, in addition to MsrA, another enzyme MsrB has a similar function, reducing sulfoxide residues; however, the substrates of MsrA and MsrB are diastereomers, methionine *S*-sulfoxide residues and methionine *R*-sulfoxide residues in protein [64]. Using *msrA/B* double mutant, the existence of other methionine sulfoxide reductases have been revealed in *E. coli* and *Saccharomyces cerevisiae* [65]. At least six different methionine sulfoxide reductases were

found in *E.coli*, which are MsrA, MsrB, fRMsr, fSMsr, MsrA1, Mem-R and S-Msr [66]. In the six enzymes, MsrA, fSMsr, MsrA1 and S-Msr have similar substrate specificity [66]. Accordingly, *msrA* in *X. fastidiosa* seems not to be a pseudogene; some other genes may have redundant roles of *msrA* in *X. fastidiosa*. In the genome of *X. fastidiosa*, *msrA* and *msrB* have been annotated in different locus; transcripts of both genes were observed in whole transcriptome analysis; and both gene are single copy. *msrA/B* double mutant of *X. fastidiosa* is necessary to be constructed for future studies, to elucidate whether other gene(s) is/are functionally similar to *msrA*.

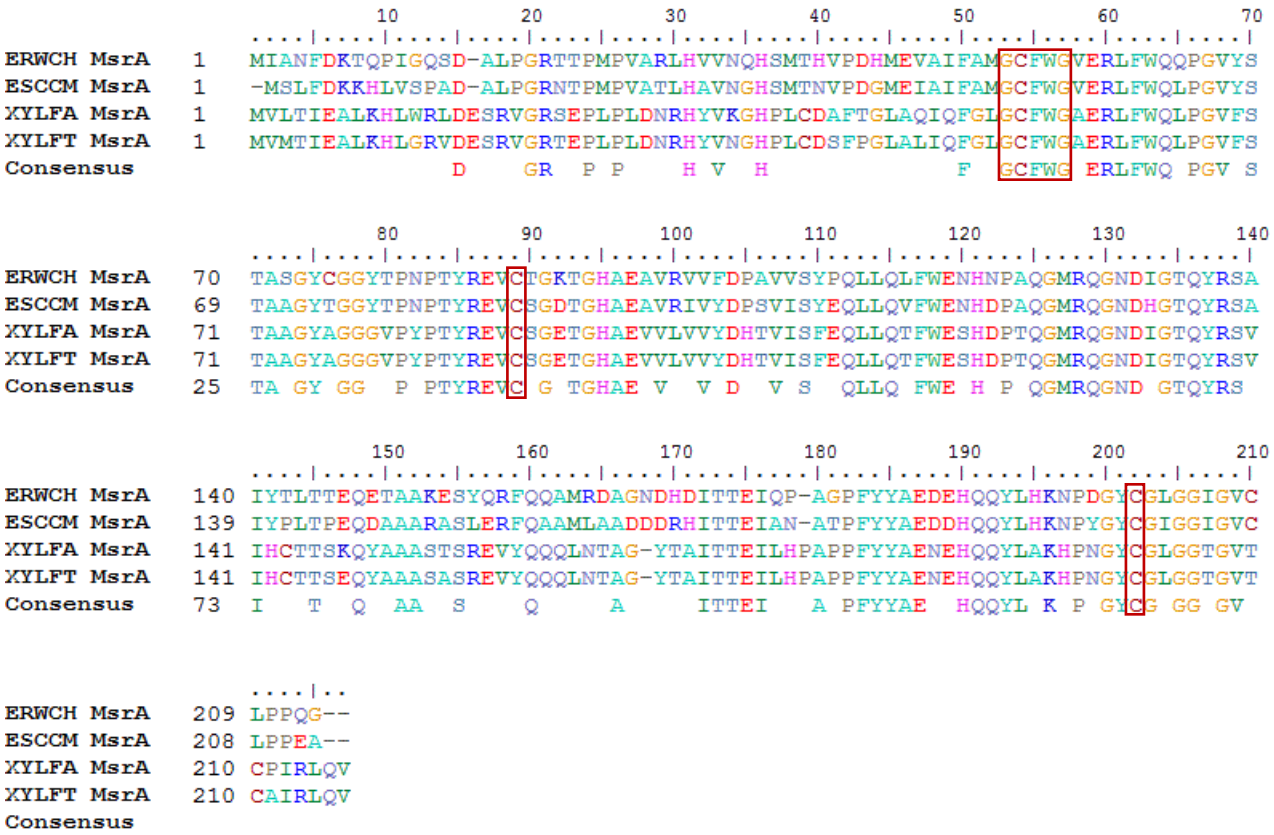

Figure 3-11. Sequence alignment of MsrA from various strains. Sequence alignment was accomplished with BioEdit (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). The putative

active site of MsrA is shown in red box, which is conserved in all strains. ERWCH, *Erwinia chrysanthemi* strain 3937 [27]; ESCCM, *Escherichia coli* strain MC1061 [56]; XYLFA, *X. fastidiosa* '9a5c'; XYLFT, *X. fastidiosa* 'Temecula'; MsrA sequence of 'WM1-1' is same to that of 'Temecula'.

In summary, although the mechanism of Ca regulated virulence traits of *X. fastidiosa* is still unclear, through evaluating the effect of *mopB* and *msrA* mutations in two *X. fastidiosa* strains, we demonstrated that outer membrane protein MopB is required for biofilm formation, motility, pilus biogenesis and virulence of *X. fastidiosa*, but *msrA* gene play no role in these processes under the conditions evaluated. Our findings in this work suggest outer membrane is associated with the virulence of *X. fastidiosa*, and outer membrane proteins are potential targets for developing new strategies to control diseases caused by *X. fastidiosa*. This work also suggests that *X. fastidiosa* probably possess multiple methionine sulfoxide reductases which are worthy to identify in future studies.

References

1. Bae, C., et al., *Infection processes of xylem-colonizing pathogenic bacteria: possible explanations for the scarcity of qualitative disease resistance genes against them in crops.* Theoretical and Applied Genetics, 2015. **128**(7): p. 1219-1229.
2. Chatterjee, S., R.P. Almeida, and S. Lindow, *Living in two worlds: the plant and insect lifestyles of Xylella fastidiosa.* Phytopathology, 2008. **46**(1): p. 243.
3. Almeida, R.P., et al., *Use of a Green Fluorescent Strain for Analysis of Xylella fastidiosa Colonization of Vitis vinifera.* Applied and Environmental Microbiology, 2003. **69**(12): p. 7319-7327.
4. Meng, Y., et al., *Upstream migration of Xylella fastidiosa via pilus-driven twitching motility.* Journal of bacteriology, 2005. **187**(16): p. 5560-5567.
5. Li, Y., et al., *Type I and type IV pili of Xylella fastidiosa affect twitching motility, biofilm formation and cell–cell aggregation.* Microbiology, 2007. **153**(3): p. 719-726.
6. Zaini, P.A., et al., *The iron stimulon of Xylella fastidiosa includes genes for type IV pilus and colicin V-like bacteriocins.* Journal of bacteriology, 2008. **190**(7): p. 2368-2378.
7. Silva-Stenico, M.E., et al., *Growth and siderophore production of Xylella fastidiosa under iron-limited conditions.* Microbiological research, 2005. **160**(4): p. 429-436.
8. Andersen, P.C., et al., *Influence of xylem fluid chemistry on planktonic growth, biofilm formation and aggregation of Xylella fastidiosa.* FEMS Microbiology Letters, 2007. **274**(2): p. 210-217.
9. Navarrete, F. and L. De La Fuente, *Response of Xylella fastidiosa to zinc: decreased culturability, increased exopolysaccharide production, and formation of resilient biofilms under flow conditions.* Applied and Environmental Microbiology, 2014. **80**(3): p. 1097-1107.
10. Cruz, L.F., P.A. Cobine, and L. De La Fuente, *Calcium increases Xylella fastidiosa surface attachment, biofilm formation, and twitching motility.* Applied and Environmental Microbiology, 2012. **78**(5): p. 1321-1331.
11. Cobine, P.A., et al., *Xylella fastidiosa differentially accumulates mineral elements in biofilm and planktonic cells.* PloS one, 2013. **8**(1): p. e54936.
12. Permyakov, E.A. and R.H. Kretsinger, *Cell signaling, beyond cytosolic calcium in eukaryotes.* Journal of inorganic biochemistry, 2009. **103**(1): p. 77-86.
13. Ikura, M., M. Osawa, and J.B. Ames, *The role of calcium‐binding proteins in the control of transcription: structure to function.* Bioessays, 2002. **24**(7): p. 625-636.
14. Domínguez, D.C., M. Guragain, and M. Patrauchan, *Calcium binding proteins and calcium signaling in prokaryotes.* Cell calcium, 2015. **57**(3): p. 151-165.
15. Bilecen, K. and F.H. Yildiz, *Identification of a calcium‐controlled negative regulatory system affecting Vibrio cholerae biofilm formation.* Environmental microbiology, 2009. **11**(8): p. 2015-2029.
16. Parker, J.K., et al., *Calcium transcriptionally regulates the biofilm machinery of Xylella fastidiosa to promote continued biofilm development in batch cultures.* Environmental microbiology, 2016.
17. Domníguez, D.C., et al., *Proteome Analysis of B. subtilis in Response to Calcium.* Journal of Analytical & Bioanalytical Techniques, 2013. 2011.

18. Patrauchan, M.A., S.A. Sarkisova, and M.J. Franklin, *Strain-specific proteome responses of Pseudomonas aeruginosa to biofilm-associated growth and to calcium.* Microbiology, 2007. **153**(11): p. 3838-3851.
19. Rigden, D.J. and M.Y. Galperin, *The DxDxDG motif for calcium binding: multiple structural contexts and implications for evolution.* Journal of molecular biology, 2004. **343**(4): p. 971-984.
20. Orans, J., et al., *Crystal structure analysis reveals Pseudomonas PilY1 as an essential calcium-dependent regulator of bacterial surface motility.* Proceedings of the National Academy of Sciences, 2010. **107**(3): p. 1065-1070.
21. Johnson, M.D., et al., *Pseudomonas aeruginosa PilY1 binds integrin in an RGD-and calcium-dependent manner.* PloS one, 2011. **6**(12): p. e29629.
22. Porsch, E.A., et al., *Calcium binding properties of the Kingella kingae PilC1 and PilC2 proteins have differential effects on type IV pilus-mediated adherence and twitching motility.* Journal of bacteriology, 2013. **195**(4): p. 886-895.
23. Cruz, L.F., et al., *Calcium-enhanced twitching motility in Xylella fastidiosa is linked to a single PilY1 homolog.* Applied and Environmental Microbiology, 2014. **80**(23): p. 7176-7185.
24. Leite, B., et al., *Genomics and X-ray microanalysis indicate that Ca^{2+} and thiols mediate the aggregation and adhesion of Xylella fastidiosa.* Brazilian Journal of Medical and Biological Research, 2002. **35**(6): p. 645-650.
25. Chen, Y.-Y., et al., *Mutation of the gene encoding a major outer-membrane protein in Xanthomonas campestris pv. campestris causes pleiotropic effects, including loss of pathogenicity.* Microbiology, 2010. **156**(9): p. 2842-2854.
26. Dandekar, A.M., et al., *An engineered innate immune defense protects grapevines from Pierce disease.* Proceedings of the National Academy of Sciences, 2012. **109**(10): p. 3721-3725.
27. Wizemann, T.M., et al., *Peptide methionine sulfoxide reductase contributes to the maintenance of adhesins in three major pathogens.* Proceedings of the National Academy of Sciences, 1996. **93**(15): p. 7985-7990.
28. Van Sluys, M., et al., *Comparative analyses of the complete genome sequences of Pierce's disease and citrus variegated chlorosis strains of Xylella fastidiosa.* Journal of bacteriology, 2003. **185**(3): p. 1018-1026.
29. Parker, J.K., J.C. Havird, and L. De La Fuente, *Differentiation of Xylella fastidiosa strains via multilocus sequence analysis of environmentally mediated genes (MLSA-E).* Applied and Environmental Microbiology, 2012. **78**(5): p. 1385-1396.
30. Oliver, J., et al., *Ionome changes in Xylella fastidiosa-infected Nicotiana tabacum correlate with virulence and discriminate between subspecies of bacterial isolates.* Molecular Plant-Microbe Interactions, 2014. **27**(10): p. 1048-1058.
31. Davis, M., A. Purcell, and S. Thomson, *Isolation media for the Pierce's disease bacterium.* Phytopathology, 1980. **70**(5): p. 425-429.
32. Davis, M., R. Whitcomb, and A. Gillaspie Jr, *Fastidious bacteria of plant vascular tissue and invertebrates (including so called rickettsia-like bacteria).* The prokaryotes: a handbook on habitats, isolation, and identification of bacteria. Berlin, 1981: p. 2172-88.
33. Davis, M.J., W.J. French, and N.W. Schaad, *Axenic culture of the bacteria associated with phony disease of peach and plum leaf scald.* Current Microbiology, 1981. **6**(5): p. 309-314.

34. Matsumoto, A. and M.M. Igo, *Species-specific type II restriction-modification system of Xylella fastidiosa Temecula1.* Applied and Environmental Microbiology, 2010. **76**(12): p. 4092-4095.
35. Francis, M., et al., *Genome-based PCR primers for specific and sensitive detection and quantification of Xylella fastidiosa.* European Journal of Plant Pathology, 2006. **115**(2): p. 203-213.
36. Doyle, J. and J. Doyle, *Genomic plant DNA preparation from fresh tissue-CTAB method.* Phytochem Bull, 1987. **19**(11): p. 11-15.
37. Kung, S.H. and R.P. Almeida, *Natural competence and recombination in the plant pathogen Xylella fastidiosa.* Applied and Environmental Microbiology, 2011. **77**(15): p. 5278-5284.
38. Guilhabert, M.R. and B.C. Kirkpatrick, *Identification of Xylella fastidiosa antivirulence genes: hemagglutinin adhesins contribute to X. fastidiosa biofilm maturation and colonization and attenuate virulence.* Molecular Plant-Microbe Interactions, 2005. **18**(8): p. 856-868.
39. Burdman, S., et al., *Aggregation in Azospirillum brasilense: effects of chemical and physical factors and involvement of extracellular components.* Microbiology, 1998. **144**(7): p. 1989-1999.
40. De La Fuente, L., et al., *Assessing adhesion forces of type I and type IV pili of Xylella fastidiosa bacteria by use of a microfluidic flow chamber.* Applied and Environmental Microbiology, 2007. 73(8): p. 2690-2696.
41. De La Fuente, L., et al., *The bacterial pathogen Xylella fastidiosa affects the leaf ionome of plant hosts during infection.* PloS one, 2013. **8**(5): p. e62945.
42. Castiblanco, L.F. and G.W. Sundin, *New insights on molecular regulation of biofilm formation in plant‐associated bacteria.* Journal of integrative plant biology, 2015.
43. Tumber, K.P., J.M. Alston, and K. Fuller, *Pierce's disease costs California $104 million per year.* California Agriculture, 2014. **68**(1-2).
44. Francis, M., E. Civerolo, and G. Bruening, *Improved bioassay of Xylella fastidiosa using Nicotiana tabacum cultivar SR1.* Plant Disease, 2008. 92(1): p. 14-20.
45. Silhavy, T.J., D. Kahne, and S. Walker, *The bacterial cell envelope.* Cold Spring Harbor perspectives in biology, 2010. **2**(5): p. a000414.
46. Ruiz, N., D. Kahne, and T.J. Silhavy, *Advances in understanding bacterial outer-membrane biogenesis.* Nature Reviews Microbiology, 2006. **4**(1): p. 57-66.
47. Koebnik, R., K.P. Locher, and P. Van Gelder, *Structure and function of bacterial outer membrane proteins: barrels in a nutshell.* Molecular microbiology, 2000. **37**(2): p. 239-253.
48. azzaq Belaaouaj, A., K.S. Kim, and S.D. Shapiro, *Degradation of outer membrane protein A in Escherichia coli killing by neutrophil elastase.* Science, 2000. **289**(5482): p. 1185-1187.
49. Van Houdt, R. and C.W. Michiels, *Role of bacterial cell surface structures in Escherichia coli biofilm formation.* Research in microbiology, 2005. **156**(5): p. 626-633.
50. Burdman, S., et al., *Involvement of type IV pili in pathogenicity of plant pathogenic bacteria.* Genes, 2011. **2**(4): p. 706-735.
51. Burrows, L.L., *Pseudomonas aeruginosa twitching motility: type IV pili in action.* Annual review of microbiology, 2012. 66: p. 493-520.

52. Voegel, T.M., et al., *Localization and characterization of Xylella fastidiosa haemagglutinin adhesins.* Microbiology, 2010. **156**(7): p. 2172-2179.
53. Mattick, J.S., *Type IV pili and twitching motility.* Annual Reviews in Microbiology, 2002. **56**(1): p. 289-314.
54. Craig, L. and J. Li, *Type IV pili: paradoxes in form and function.* Current opinion in structural biology, 2008. **18**(2): p. 267-277.
55. Das, T., et al., *Influence of calcium in extracellular DNA mediated bacterial aggregation and biofilm formation.* PloS one, 2014. **9**(3): p. e91935.
56. El Hassouni, M., et al., *The minimal gene set member msrA, encoding peptide methionine sulfoxide reductase, is a virulence determinant of the plant pathogen Erwinia chrysanthemi.* Proceedings of the National Academy of Sciences, 1999. **96**(3): p. 887-892.
57. Dhandayuthapani, S., et al., *Peptide methionine sulfoxide reductase (MsrA) is a virulence determinant in Mycoplasma genitalium.* Journal of bacteriology, 2001. **183**(19): p. 5645-5650.
58. Douglas, T., et al., *Methionine sulfoxide reductase A (MsrA) deficiency affects the survival of Mycobacterium smegmatis within macrophages.* Journal of bacteriology, 2004. **186**(11): p. 3590-3598.
59. Mintz, K.P., et al., *Peptide methionine sulfoxide reductase (MsrA) is not a major virulence determinant for the oral pathogen Actinobacillus actinomycetemcomitansa.* Microbiology, 2002. **148**(11): p. 3695-3703.
60. de Souza, A.A., et al., *Analysis of gene expression in two growth states of Xylella fastidiosa and its relationship with pathogenicity.* Molecular Plant-Microbe Interactions, 2003. **16**(10): p. 867-875.
61. de Souza, A.A., et al., *Expression of pathogenicity-related genes of Xylella fastidiosa in vitro and in planta.* Current Microbiology, 2005. **50**(4): p. 223-228.
62. Moskovitz, J., et al., *Identification and characterization of a putative active site for peptide methionine sulfoxide reductase (MsrA) and its substrate stereospecificity.* Journal of Biological Chemistry, 2000. **275**(19): p. 14167-14172.
63. Weissbach, H., et al., *Peptide methionine sulfoxide reductase: structure, mechanism of action, and biological function.* Archives of Biochemistry and Biophysics, 2002. **397**(2): p. 172-178.
64. Delaye, L., et al., *Molecular evolution of peptide methionine sulfoxide reductases (MsrA and MsrB): on the early development of a mechanism that protects against oxidative damage.* Journal of Molecular Evolution, 2007. **64**(1): p. 15-32.
65. Lin, Z., et al., *Free methionine-(R)-sulfoxide reductase from Escherichia coli reveals a new GAF domain function.* Proceedings of the National Academy of Sciences, 2007. **104**(23): p. 9597-9602.
66. Weissbach, H., L. Resnick, and N. Brot, *Methionine sulfoxide reductases: history and cellular role in protecting against oxidative damage.* Biochimica et Biophysica Acta (BBA)-Proteins and Proteomics, 2005. **1703**(2): p. 203-212.