

The role of calcium in the regulation of *Xylella fastidiosa* virulence

by

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A dissertation submitted to the Graduate Faculty of
Auburn University
in partial fulfillment of the
requirements for the Degree of
Doctor of Philosophy in Plant Pathology

Auburn, Alabama
December 14, 2013

Keywords: *Xylella fastidiosa*, calcium, cells aggregation, biofilm, twitching motility,
pilYI

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Abstract

The Gram-negative plant pathogenic bacterium *Xylella fastidiosa* inhabits the xylem vessels of the host, where it forms biofilms thought to be responsible for disease symptoms. Even though knowledge regarding the mechanism of pathogenicity is still inconclusive, symptoms depend on the ability of the bacterium to spread, colonize, and form biofilms. I hypothesize that sap mineral components have a role in the bacterial pathogenicity and virulence by promoting adhesion, aggregation, and twitching motility. The general objective of this research was to establish the role of the specific mineral element calcium (Ca) in the virulence traits of *X. fastidiosa*. A positive correlation between the increase in the concentration of Ca and the formation of biofilm and twitching motility was observed. The addition of Ca significantly increased the adhesion force to surfaces and autoaggregation and promoted the up-regulation of genes involved in the production of exopolysaccharide in biofilm cells. Further experiments were conducted to understand the molecular mechanisms of Ca-regulated twitching motility. Expression analyses of the type IV pili genes *pilA*, *pilT*, *pilB*, and the three *pilYI* homologs (PD0023, PD0052, PD1611) were performed on cells incubated in microfluidic chambers under replete and depleted Ca conditions. Up-regulation was found for *pilT*, encoding the retraction ATPase in charge of pili depolymerization, and one of the *pilYI* homologs (PD1611), encoding a protein located at the tip of the pili. Sequence analysis identified a Ca-binding domain only in the up-regulated *pilYI*-PD1611 homolog. The deletion mutagenesis of *pilYI*-PD1611 results in reduced motility and a Ca-blinded phenotype, whereas twitching motility of a *pilYI* PD0023

mutant was not affected by Ca supplementation. Results indicate that the mechanism of Ca-dependent twitching motility is associated with Ca-binding by only one of the *pilYI* homologues found in *X. fastidiosa*. *In planta* experiments using the system model *Nicotina tabaccum* were carried out to determine the effect of Ca on *X. fastidiosa* infection. Results indicated that Ca supplementation increases bacterial populations and intensifies disease symptoms. In turn, bacterial infection elicits an increase in the accumulation of Ca in the plant above the levels caused by the fertilization with Ca. Additionally, a general change in the mineral composition of the infected plants was observed compared with the non-infected plants. The results of this research suggest that Ca acts as a key regulator of *X. fastidiosa* virulence.

Acknowledgement

I would like to express my deepest gratitude to my advisor Leonardo De La Fuente for the continuous support of my Ph.D study and research, for his patience, motivation, enthusiasm and good energy. His guidance helped me in all the time of research. I could not have imagined having a better advisor and mentor for my Ph.D study.

Besides my advisor, I would like to thanks the rest of the committee members Dr Koeppler, Dr Lawrence and specially Dr. Paul Cobine for his comments and suggestions for the development of the project; In addition I want to thank the outside reader Dr. Arias

I would like to thanks Jennifer Parker for making my life easier in all the aspects, and for trying to keep the lab as a clean and organized, and overall to be there for all of the ups and downs of my research and life.

I thanks Fernando Navarrete for asking me question that nobody cares, deleting my document from the microscope computer, for criticizing my work, for his excellent suggestion and help with the experiments overall for being the “happiness” of the lab.

I thanks my labmates: Jonathan Oliver for his help with the ICP, Hongyu Chen and Prem Kandel for his help with the collection, processing of plant samples and mutational process.

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List of Abbreviations

| | |
|---------|--|
| BAPTA | 1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid |
| CFU | colony-forming unit |
| CTAB | cetyltrimethylammonium bromide |
| DSF | diffusible signal factor |
| EGTA | ethylene glycol tetraacetic acid |
| EPS | exopolysaccharide |
| ICP-OES | inductively coupled plasma – optical emission spectrometry |
| LSCM | laser scanning confocal microscopy |
| PCR | polymerase chain reaction |
| PD | Pierce's disease |
| qPCR | quantitative polymerase chain reaction |
| WT | wild type |

Chapter 1 Overview of *Xylella fastidiosa* biology and metals effect on bacterial virulence

1. The plant pathogen *Xylella fastidiosa*

Xylella fastidiosa is a gram negative plant pathogenic bacterium that exclusively inhabits the xylem vessels of the host plant (Davis et al., 1981). Bacterial cells are rod shaped with dimensions of 0.25 to 0.35 μm in radius and 0.9 to 3.5 μm in length (Wells et al., 1987). The bacterium is vector-transmitted to the host plants by various insects commonly known as sharpshooters from the families Cicadellidae, Cercopidae, Machaerotidae, and Cicadidae (Almeida et al., 2005).

The bacterium is endemic to California, where Pierce's disease was first described (Pierce 1892), as well as the Gulf Coast. There is no effective control for the infection of *X. fastidiosa*. Disease management practices are focused on control of the vectors and the deployment of tolerant or resistant varieties (Hopkins and Purcell, 2002).

1.1 Host Range and Geographic Distribution

Different strains of *X. fastidiosa* infect a wide host range. Classification of the strains of *X. fastidiosa* resulting from DNA similarity assays and ITS sequence divide *X. fastidiosa* into four subspecies: *X. fastidiosa* subsp. *fastidiosa* (grape, alfalfa, almond and maple), *X. fastidiosa*

subsp. *multiplex* (peach, plum, sycamore, elm, and almond), and *X. fastidiosa* subsp. *pauca* (citrus) *X. fastidiosa* subsp. *sandyi*. (oleanders) (Schaad et al., 2004; Schuenzel et al., 2005). The most economically important diseases are Pierce's disease (PD) of grapevines and citrus variegated chlorosis (CVC). Other crops such as alfalfa, peach, plum, almond, and coffee and many forest trees and landscape plants including oak, elm, maple, and oleander are also attacked by the bacterium. *Xylella fastidiosa* infections have been reported in United States (Alabama, Arizona, California, Florida, Georgia, Louisiana, Mississippi, Missouri, Montana, North Carolina, South Carolina, Texas), Mexico, Costa Rica, Argentina, and Brazil (Hopkins and Purcell, 2002).

1.2 Disease Development

The distinctive disease symptoms of *X. fastidiosa* infection include marginal leaf scorch, green islands on stems, abscission of leaves leaving the petiole attached to the stem, uneven maturation of the periderm, fruit desiccation, and finally plant death (Stevenson et al., 2005). Symptoms have been associated with water deficits caused by xylem blockage due to the formation of bacterial aggregates during host colonization and products of plant defense response such as gums and tyloses (projections of parenchyma cells walls into xylem treachery elements) (Stevenson et al., 2004).

Vessel blockage that leads to water deficit is the most accepted hypothesis as the cause of disease symptoms (Hopkins, 1989). However, this hypothesis has been subject to controversy due to the lack of consistency in findings that support it. For instance, studies have shown no correlation

between the quantity of bacteria and the expression of symptoms or between the proportion of vessel colonized and blocked in symptomatic leaves (<40%) (Newman, 2003; Gambetta et al., 2007). Other studies found no correlation between the loss of vascular function caused by the disease and the development of symptoms or between the disease symptoms and water deficit, suggesting that leaf scorching may be independent of vessels occlusion and decrease of water conductance (Thorne et al., 2006; Choat et al., 2009).

Once the bacterium is injected by the vector into the host, the exact mechanism of pathogenesis is unknown. However, disease development depends on the ability of the bacterium to colonize and multiply in the plant (Chatterjee et al., 2008a). The movement of the bacterium is limited by pit membranes separating neighboring vessels. Due to the size of the pores, which are smaller in diameter than the bacterial cells, the systemic spread of the bacterium requires an active mechanism. Evidence of the presence of a gene encoding polygalacturonase (an extracellular degrading enzyme) and the requirement of polygalacturonase for bacterial spread was found (Roper et al., 2005). Additionally, an increase in pit membrane pore size in the presence of polygalacturonase and β -glucanase, both encoded by *X. fastidiosa*, was determined by Perez-Donoso et al. (2010).

The progression of bacterial colonization has been described as having three main steps that lead to the development of symptoms: i) cell attachment and initial colonization of the stem, ii) formation of biofilm and associated gums in petioles and leaves, iii) formation of tyloses in stems, iv) radial spread of bacteria through the pit membranes (Newman, 2003; Stevenson et al., 2005).

1.3 Molecular Disease Determinants

Cell attachment and biofilm formation are considered determinants for plant infection. Biofilm is used by the bacteria to protect the community of cells from antimicrobials, host defense compounds, and dehydration to promote cell-to-cell interaction for exchanging of molecule signals and to optimize nutrient uptake from the flow of xylem sap by the formation of exopolysaccharides (EPS) (Hall-Stoodley et al., 2004). *Xylella fastidiosa* encodes various surface-associated proteins that are thought to be important for intra-plant migration, host colonization, and biofilm formation. For instance, fimbrial proteins are implicated in the production of type I and type IV pili, both located on one pole of the cell (Meng et al., 2005). These structures have a role in twitching motility and cell attachment to the surface. The type I pilus is responsible for cell anchorage, and the type IV pilus extends and retracts to assist cell motility (De La Fuente et al., 2007; Li et al., 2007).

Afimbrial adhesins are surface proteins that produce projections in the outer membrane. This group of adhesins includes hemmagglutinins, which have been identified in a set of transposon mutants that exhibited hypervirulence, faster doubling time, an increase in movement through the plant, and altered cell-to-cell attachment and biofilm maturation (Guilhabert and Kirkpatrick, 2005). Expression analyses of genes encoding fimbrial and afimbrial adhesins have shown differential regulation temporally and spatially during host colonization and biofilm formation (De Souza et al., 2005; Caserta et al., 2010).

As in other species of bacteria, quorum sensing plays a role in pathogenicity in a cell density-dependent manner. *Xylella fastidiosa* harbors a cell-to-cell signaling system mediated by a small molecule called diffusible signal factor (DSF), encoded by the *rff* cluster of genes that regulates bacterial aggregation, attachment, biofilm formation, insect transmission, and virulence (Chatterjee et al., 2008b; Chatterjee et al., 2008a).

1.4 Role of Nutrients in Bacterial Biology

Essential nutrients are required for many cellular functions in eukaryotic and prokaryotic cells. Trace elements, for instance, are required for diversity of metalloproteins and as cofactors in enzymatic reactions (Waldron and Robinson, 2009). Bacterial growth is usually limited by the availability of necessary nutrients. Different environments vary in the concentrations of solutes, so bacteria have evolved mechanisms for regulating cellular nutrient concentrations (Snyder and Champness, 2003).

Iron plays an important role as a regulator of pathogenicity-related genes and host bacterial interactions (Mila et al., 1996). Iron is essential for the activity of several enzymes acting in electron transport processes (Expert, 2007). Production of siderophores (low molecular weight molecules that possess an affinity for Fe) has received special attention for its role in plant pathogen interactions. The presence of genes encoding receptors for siderophores and proteins involved in iron uptake suggests the importance of iron for the growth of the bacteria in the xylem and virulence (Silva-Stenico et al., 2005; Pacheco et al., 2006). Furthermore, whole-genome transcription analyses of *X. fastidiosa* under high and low iron concentrations establish

the role of this metal in the regulation of genes required for type I and type IV pili, as well as for genes involved in the production and the synthesis of a diffusible signal factor and colicin V, a toxin that acts against other bacterium and improves *X. fastidiosa* survival during host colonization (Zaini et al., 2008).

Copper is an essential micronutrient metal for both plants and plant pathogens (Evans and Solberg, 2007). It is recognized for its effects as a biocide and has been associated with denaturation of nucleic acids, inhibition of protein activity, and changes in plasma membrane permeabilization (Borkow and Gabbay, 2005). Copper homeostasis seems to play an important role in bacteria survival (Rouch and Brown, 1997). Rodrigues et al. (2008) investigated the effects of copper on biofilm and planktonic cells and on the expression of gene-encoding proteins (Rodrigues et al., 2008). Results indicated that cells in biofilm were more resistant to copper than those in suspension. The resistance of biofilm cells seems to be determined by the amount of EPS present in the biofilm and its ability to bind metal cations as well as the presence of molecular pumps in charge of cation extrusion.

Leite et al. (2002) examined *X. fastidiosa* biofilm grown *in planta* and, using x-ray microanalyses, determined the presence of Ca, Mg, and sulfur peaks occluded xylem vessels. This result allowed the researchers to propose a model where Ca and Mg work as a bridge between the negatively-charged xylem vessels and the negatively-charged bacterial exopolysaccharides (Leite et al., 2002). In addition to the effect of electrostatic interactions, there is some evidence of the association of calcium with the modulation of prokaryotic cellular processes including nucleoid structure, protein phosphorylation, and alteration in the distribution

of membrane lipids (Norris et al., 1996). Furthermore, there is a widespread presence of calmodulin-like proteins, which show affinity for calcium, and channels and transporters for calcium regulation in prokaryotic cells (Michiels et al., 2002).

The effect of the xylem-fluid chemistry on bacterial growth has been analyzed. Studies showed the determinant role in bacteria of xylem sap compounds in bacterial growth, the ability of the cells to attach to the surface and to each other, and in biofilm formation. For example, Andersen et al. (2007) studied the effect of different sap components including inorganic ions (Ca, Mg, P, Zn, and Cu) of susceptible and resistant plants. The findings indicated a correlation between the levels of zinc and copper and the amount of planktonic growth and a correlation between calcium levels and the amount of biofilm formation (Andersen et al., 2007).

Xylella fastidiosa infection symptoms resemble nutritional imbalances in the host. These imbalances have been thought to be caused by deficient water supply or by mechanisms related to bacterial mineral sequestration. Analysis of foliar areas of citrus infected with *X. fastidiosa* showed deficiencies of P, K, and Zn and high concentrations of Fe and Mn (Silva-Stenico et al., 2009).

2 The influence of Ca on bacterial virulence

2.1 Calcium has structural and regulatory functions

Calcium is the fifth most abundant element in the Earth's crust, with an average concentration of 3.64% (Datnoff et al., 2007). This divalent alkaline cation is one of the most abundant components of plant and animal fluids and tissues, where it is utilized for structural and

regulatory functions (Hepler, 2005). Specific characteristics of this element, such as its flexibility in exhibiting different coordination numbers (points of attachment) and its ability to form complexes with proteins, membranes, and organic acids, make Ca an important structural component of macromolecules, forming intermolecular linkages (Kundla et al., 2010). The function of Ca as a regulatory molecule has been associated with its low cytosolic concentration and the cellular capacity to increase its concentration in response to environmental signals. Ca gradients are the base of mechanisms that transmit information into cells, transducing signals to target different calcium-binding proteins (Jaiswal, 2001; Shemarova and Nesterov, 2005)

2.2 Acquisition and distribution by plants

Plants absorb Ca from the soil solution as divalent cations. Calcium accessibility for plant uptake depends on several factors including soil pH (optimal range is 4.5-6.0), soil mineral composition, and the presence of other elements (Mg, K and, ammonium) (Datnoff et al., 2007). Calcium is moved through plant xylem vessels by the transpiration stream and is relatively immobile in the phloem. Calcium tends to accumulate in leaves and in older parts of the plant, and leaves are the organs with the highest Ca content in the plant. At the cellular level, Ca is mainly located in the cell wall bound as pectates in the middle lamella; this characteristic is important for cell wall stabilization and cation exchange (Marschner, 1995). In the cytosol, Ca is present in very low concentrations, mainly stored in the vacuoles and the endoplasmic reticulum. Constitutive permeability of the membranes and membrane transporters maintain optimal Ca concentration in the cytosol and avoid cytotoxicity (Conn et al., 2011).

2.3 Roles of calcium in bacterial metabolism

Studies of diverse prokaryotic cellular processes have linked Ca with varied regulatory functions including cell cycle, cell division, phospholipid synthesis and configuration, nucleoid structure, protein phosphorylation, and alteration in the distribution of membrane lipids (Norris et al., 1996; Yu and Margolin, 1997; Onoda et al., 2000; Dominguez, 2004). In addition, Ca plays structural roles by preserving the integrity and stability of the lipopolysaccharide layer and the cell wall (Smith, 1995).

Multiple Ca-binding proteins have been described in bacteria as having varied functions, and they are also implicated in Ca homeostasis. The most studied type of Ca-binding proteins is the calmodulins (calcium modulated proteins) that were initially described in eukaryotic organisms (Gifford et al., 2007). Calmodulins are acidic proteins of small size (15-22KDa) that contain a typical helix-loop-helix motif called EF-hand, which is in charge of Ca binding. In bacteria, calmodulin-like proteins have been recently identified, and they are believed to play a significant role in cellular regulation as in their eukaryotic counterparts (Michiels et al., 2002). Some of the bacterial calmodulin-like proteins exhibit a similar structure to the eukaryotic ones, though some variation in the number of EF-hand motifs and their organization has also been observed (Michiels et al., 2002; Zhou et al., 2006). The functions of the proteins holding the EF-hand binding motives are diverse and include proteins related to buffering and transport of Ca, extracellular degrading enzymes, galactose, glucose and ion transport, chemotaxis, protein metabolism, DNA modification, and stress response (Michiels et al., 2002). Another known Ca-binding motif in bacteria is the haemolysin-type, also called the repeats in toxin (RTX) motif. This motif is common to proteins secreted by the type I secretion system of Gram-negative

bacteria (Linhartová et al., 2010). RTX-containing proteins are characterized by the presence of tandem repeats of nine conserved residues rich in glycine and aspartame, located in the C-terminal of the protein. Ca ions bind to the C-terminal once the protein is secreted, changing the conformation of the protein to secondary structure and then to tertiary structure to make it functional. RTX proteins have a broad spectrum of functions including as toxins, proteases, lipases, bacteriocins, and nodulation proteins (Linhartová et al., 2010).

2.4 Regulation of calcium homeostasis in bacteria

Prokaryotic and eukaryotic cells require an extrusion system to maintain tolerable levels of Ca (Hepler, 2005; Case et al., 2007). High intracellular Ca concentrations have deleterious effects on the cells, including proteins and nucleic acids aggregation and phosphate precipitation (Campbell, 1990). In bacteria, intracellular concentrations of Ca range from 90nM to 300nM, which is 1000 times lower than in the extracellular medium (Gangola and Rosen, 1987; Norris et al., 1996; Torrecilla et al., 2000). Some authors suggest that the evolution of Ca homeostatic systems was the base for the later use of Ca as a regulatory element (Jaiswal, 2001; Shemarova and Nesterov, 2005). The prerequisite for the function of Ca as a regulatory molecule is the presence of mechanisms that control its cellular concentration (Hofer and Lefkimmiatis, 2007). The regulation of free Ca in the bacterial intracellular space is carried out by primary and secondary active transport (ion channels for influx and energy dependent pumps and antiporters for efflux) (Norris et al., 1996; Paulsen et al., 2000; Shemarova and Nesterov, 2005).

Voltage operated calcium channels (VOCCs) are the only mechanism for Ca influx reported in bacteria. VOCCs are a class of transmembrane Ca-selective channels that are activated by membrane potential change, have affinity for divalent ions (Ca, strontium, and barium), and are

blocked by lanthanum (Van Petegem and Minor, 2006; Fernandez-Morales et al., 2011). Two types of VOCCs have been found in bacteria. The first type, reported in *Bacillus halodurans* (Durell and Guy, 2001), is composed of multiple protein subunits (Koishi et al., 2004). The second type of VOCC, reported in *E. coli* and *Bacillus subtilis* (Matsushita et al., 1989), is composed of a lipidic polymer (poly-3-hydroxybutyrate) and inorganic polyphosphate (Reusch et al., 1995).

The extrusion of Ca from bacterial cells depends on two mechanisms: ATPase Ca pumps and antiporters (Norris et al., 1996; Dominguez, 2004; Shemarova and Nesterov, 2005). ATPase Ca pumps are believed to be the main mechanism of calcium efflux in bacteria (Martins et al., 2011). They show high similarity with the eukaryotic P-type ATPase pumps (Gambel et al., 1992; Desrosiers et al., 1993), which are composed of two polypeptide subunits: α (the bigger subunit) and β (the smaller one). The small subunit is characterized by having a transmembrane domain and an ATP binding site (Lodish, 2000). ATPase Ca pumps have been identified in the cyanobacterium *Synechococcus* sp. (Kanamaru et al., 1993; Berkelman et al., 1994), the soil bacterium *Flavobacterium odoratum* (Gambel et al., 1992), the soil/plant- associated bacterium *B. subtilis* (Raeymaekers et al., 2002), and the human pathogen *Streptococcus pneumoniae* (Trombe, 1999; Rosch et al., 2008). Antiporters use proton or sodium (Na^+) electrochemical gradients to remove Ca from the cell. For this, Na^+ flows down its gradient across the plasma membrane to the interior of the cells, and the energy released from this process is used to transport Ca against the gradient (Slonczewski et al., 2009). Antiporters are characterized by the presence of a transmembrane domain with about 400 amino acid residues that contain a central hydrophobic motif with a high content of acidic amino acids (Waditee et al., 2004). In *E. coli*,

the protein ChaA catalyzes $\text{Ca}^{2+}/\text{H}^{+}$ and $\text{Na}^{+}/\text{H}^{+}$ exchange reactions at alkaline pH (Ivey et al., 1993; Ohyama et al., 1994). In *S. pneumoniae*, a $\text{Na}^{+}/\text{Ca}^{2+}$ antiporter was found to participate in both Ca influx and efflux (Trombe et al., 1994).

2.5 Role of calcium in bacterial virulence

During the infection process, bacterial pathogens require the use of a collection of genetically encoded virulence determinants, which are independently regulated in response to environmental signals (Wilson et al., 2002). Calcium acts in bacterial pathogenesis, working as a signal and regulator of different pathogenicity determinants (Dominguez, 2004).

2.5.1 Extracellular enzymes

Genes encoding extracellular enzymes can be induced constitutively or as a response to environmental stimuli (Agrios, 2005). In the bacterium *Pectobacterium carotovorum*, causal agent of soft rot, Ca is associated with the regulation of the extracellular hydrolytic enzymes polygalacturonase (PG), required in early stages of disease development, and pectate lyase (Pel), detected in later stage of tissue maceration (Pagel and Heitefuss, 1990; Barras et al., 1994). An increase in the extracellular Ca concentration represses the expression of the *pehA* gene encoding PG production, while the expression of *pelB* encoding the pectate lyase is enhanced (Pagel and Heitefuss, 1990; Flego et al., 1997). In *Pseudomonas aeruginosa* FRD1, the up-regulation of three extracellular proteases (alkaline protease, elastase, and PrpL protease) was demonstrated in the presence of Ca (Sarkisova et al., 2005). In other cases, Ca-binding by specific proteins is known to confer enzyme stability. The binding of Ca to specific Ca-binding motifs in enzymes

prevents polypeptide unfolding, resulting in greater resistance to denaturation and proteolysis (Nardini et al., 2000). This effect has been observed in *Clostridium thermocellum* endoglucanase-D, *Bacillus* sp. subtilisin protease, and *P. aeruginosa* PAO1 lipases (Chauvaux et al., 1990; Smith et al., 1999; Nardini et al., 2000).

2.5.2 Cell attachment

Bacterial attachment is the initial step that leads to plant host invasion, colonization, and formation of biofilms (Reed and Williams Jr, 1978; Rodríguez-Navarro et al., 2007). There is evidence that Ca is required in the function of extracellular proteins essential for the attachment of bacteria to surfaces. The biocontrol strain of *Pseudomonas putida* KT244 encodes a Ca-binding surface protein, designated LapA (large adhesion protein A), that is required for its adhesion to the seed surface (Smit et al., 1989; Espinosa-Urgel et al., 2000; Patrauchan et al., 2005). Protein analyses determined the existence of an ABC transporter for its secretion and the presence of a RTX calcium-binding domain (Hinsa et al., 2003). Similarly, Cah (calcium-binding and heat-extractable autotransporter protein), a RTX-containing protein, is implicated in cell aggregation and biofilm formation in *E. coli* (Torres et al., 2002). Rhicadhesin, another protein containing a Ca-binding motif, is required for cell attachment in *Rhizobium* spp. and in *Agrobacterium tumefaciens*. This protein contains one domain that is anchored to the bacterial cell surface in a Ca-dependent manner and a second domain that binds to the plant receptor (Smit et al., 1989; Swart et al., 1994).

In addition, Ca acts as cross-bridging between cells and substrate and stabilizer of the biofilm matrix (Fletcher, 1988; Rose, 2000; Leite et al., 2002). Leite et al. (2002) examined the *Xylella fastidiosa* biofilm *in planta* and proposed a model where Ca and Mg work as a bridge between the negatively-charged xylem vessels and the negatively-charged bacterial exopolysaccharides (Leite et al., 2002). In addition to its electrostatic effect in the accumulation of *X. fastidiosa* biofilms, evidence indicated that type I pili is implicated in the response to Ca increasing cell attachment (Cruz et al., 2012 Chapter 1).

2.5.3 Extracellular matrix production

Ca has been shown to influence the accumulation of biofilm biomass and the production of higher amounts of exopolysaccharide (EPS). This has been observed in the marine bacterium *Pseudoalteromonas* sp. (Patrauchan et al., 2005) and in *P. aeruginosa* FRD1, where Ca is responsible for the increase in the expression of *alg* operon that leads to biosynthesis of alginate, the main constituent of the bacterial extracellular matrix (Patrauchan et al., 2007). In contrast, Ca negatively affects accumulation of biofilm by *Vibrio cholerae* by decreasing the transcription of the positive biofilm regulators VpsT (for *Vibrio* polysaccharide) and VpsR (Yildiz et al., 2001). The negative regulation is imposed by the two component system Ca-R,S (the Ca sensor and the negative regulator) (Bilecen and Yildiz, 2009). Ca-inhibition of biofilm was also observed in *Staphylococcus aureus*, a commensal but sometimes pathogenic member of human skin flora. The presence of a Ca-binding motif in the surface protein Bap is responsible for a conformational change that renders the protein unable to accomplish its function in cellular auto-aggregation (Cucarella et al., 2001; Arrizubieta et al., 2004).

2.5.4 Bacterial movement and chemotaxis

Bacteria use a variety of mechanisms to move in response to environmental stimuli, escape from unfavorable conditions, and access optimal colonization sites and nutrient sources (Ottemann and Miller, 1997). Among other functions, flagella assist bacterial locomotion and also have a role in bacterial adhesion in several species (Moens and Vanderleyden, 1996). Calcium has been associated with the structure of the flagella of the nitrogen-fixing bacterium *Rhizobium meliloti*. Divalent ions, including Ca, appear to have a function in the polymerization and stability of flagella subunits, providing ionic bridges between them (Robinson et al., 1992). In magnetotactic bacteria (a marine group of Gram-negative bacteria characterized by the production of intracellular crystals of iron oxide and/or iron sulphide that lead their orientation following geomagnetic field lines) (Bazylinski and Frankel, 2004), Ca ions have been linked with the structural integrity of the sheath surrounding the flagella. The sheath consists of a glycoprotein with homology to other bacterial adhesins and to the eukaryotic Ca-binding protein cadherin (Lefèvre et al., 2010). Another structure involved in bacterial movement is the type IV pilus. Functions of type IV pili include cell adhesion, cell signaling, biofilm formation, virulence, and twitching motility (Burdman et al., 2011). In *P. aeruginosa*, Ca is required for the regulation of the motility system. PilY1, a protein located at the tip of the pilus, bears a Ca-binding motif. Binding and release of Ca elicit extension and retraction of the type IV pili for twitching motility. Ca-binding inhibits PilT-mediated pilus retraction, whereas Ca release allows retraction (Orans et al., 2009). In the plant pathogen *X. fastidiosa*, in which twitching motility is also

facilitated by type IV pili, increase in Ca concentration was correlated with the increase in cells' twitching motility speed (Cruz et al., 2012 Chapter 1).

Calcium has also been implicated in chemotaxis. In *B. subtilis* and *E. coli*, changes in the intracellular concentration of Ca are associated with changes in the flagella rotation: clockwise (tumbling in response to repellants) and swimming (counter-clockwise in response to attractants) (Ordal, 1977; Tisa and Adler, 1995; Watkins et al., 1995). Repellents caused an increase (50-150 nM) in free intracellular Ca; whereas attractants caused a small decrease in intracellular free Ca. Mutational analysis indicates that the induction of tumbling by Ca increases required the presence of Che chemosensory system proteins. The authors hypothesized that Ca may help to maintain the phosphorylated state of CheY, a protein requirement for tumbling (Tisa and Adler, 1992).

2.5.5 Secretion systems

The type III secretion system constitutes one of the most complex mechanisms by which bacteria are able to deliver virulence factors and cause disease in the host (Galan and Collmer, 1999). The type III secretion system comprises the delivery machinery, the effectors (virulence factors), and the translators (responsible for the delivery of the effectors into the host cytoplasm) (Galan and Wolf-Watz, 2006). A sensor/regulatory mechanism is required to ensure precise delivery of effectors to the target cell and prevent their unnecessary release to the extracellular milieu (Muller et al., 2001; Galan and Wolf-Watz, 2006). Ca is believed to be involved in the regulatory mechanism in some bacterial species (Straley et al., 1993). *Yersinia pestis*, *Y.*

pseudotuberculosis, and *Y. enterocolitica* contain a 70KB plasmid that controls virulence and mediates the low-calcium response (LCR). This phenomenon activates the type III secretion system and occurs at 37 °C under depleted Ca conditions *in vitro*. The response is characterized by a stop in vegetative growth and the expression of the proteins encoding the type III secretion apparatus, the effector proteins, and proteins involved in the crossing of other proteins through the host plasma membrane Yops (Yersinia outer proteins) (Portnoy et al., 1981; He, 1998). The molecular mechanisms involved in Ca sensing for the regulation of the *Yersinia* spp. type III secretion system are unknown. However, extensive research has found some of the proteins involved in the bacterial response that includes LcrQ (low calcium response protein Q) (Rimpilainen et al., 1992) and YopD (Hakansson et al., 1993), which act as negative regulators, and a protein complex, YopN- SycN-YscB-TyeA, that prevents the secretion of effector proteins in the presence of Ca. Researchers hypothesized the existence of a hierarchy in the secretion of proteins needed for the proper function of the contact-induced translocation machinery (Francis et al., 2001). A similar mechanism governing the type III secretion, controlling the secretion of effectors and translocators via Ca concentration, has been described for *Pseudomonas aeruginosa* (Frithz-Lindsten et al., 1998; Dasgupta et al., 2006), *Chlamydophila pneumonia*, human enteropathogenic and enterohemorrhagic *E. coli*, and *Citrobacter rodentium*. They all share a pathogenicity island called the locus of enterocyte effacement, encoding their type III secretion system (Deng et al., 2005).

A function of Ca has also been established in the type I secretion system of Gram-negative bacteria. A common characteristic to most of the proteins secreted through this system is the presence of the RTX motif, which is located upstream of the C-terminal secretion signal

(Linhartová et al., 2010). Binding of Ca at millimolar concentrations, once the protein is exported, causes a modification in the conformation of the RTX protein to secondary and tertiary structure (Chenal et al., 2009). These proteins conformational changes are required for their secretion through the narrow channel of the type I secretion system. Once in the outside environment, at higher Ca concentration, the binding of Ca shifts the protein conformation to make it stable for its proper biological function (Chenal et al., 2009).

Table 1-1 Summary of studies on plant pathogenic bacteria where Ca has shown to have a role in virulence

| Virulence trait | Bacterial species | Direct or indirect metal effect | Reference |
|--|--|---|---|
| Extracellular enzymes | <i>Pectobacterium carotovorum</i> | Down-regulation of <i>pehA</i> encoding polygalacturonase and up-regulation of <i>pelB</i> encoding pectate lyase. | Pagel and Heitefuss, 1990; Flego et al., 1997 Sarkisova et al., 2005; Nardini et al., 2000 Leite et al., 2002 |
| | <i>Pseudomonas aeruginosa</i> ¹ | Up-regulation genes encoding for alkaline protease, elastase, PrpL protease, and lipase. | |
| Biofilm formation | <i>Xylella fastidiosa</i> | Accumulation in biofilms <i>in planta</i> . Proposed electrostatic role in cell-to-cell or cell-surface interaction. Increase biofilm formation, adhesion to surfaces and cell-to-cell aggregation. Dependent on metabolic activity. | Cruz et al., 2012 Chapter 1 Patrauchan et al., 2007 |
| | <i>Pseudomonas aeruginosa</i> | Up-regulation of genes encoding alginate biosynthesis, results in high accumulation of biofilm. | |
| Cell attachment Twitching movement | <i>Agrobacterium tumefaciens</i> | Rhcadhesin (Ca-binding protein) involved in ion-anchoring the protein to the bacterial cell. | Swart et al., 1994 Orans et al., 2009 Cruz et al., 2012 Chapter 1 |
| | <i>Pseudomonas aeruginosa</i> <i>Xylella fastidiosa</i> | Ca-binding to type IV pili PilY1 protein elicits twitching motility. Increase speed of twitching movement. | |
| Proteins secreted by Type I secretion system | Gram-negative bacteria | Binding of Ca shifts the protein conformation, modulating its functionality. | Linhartová et al., 2010 |
| Type III secretion system | <i>Pseudomonas aeruginosa</i> | Low calcium response (LCR). Ca depletion elicits the expression of the type III secretion apparatus and the effector proteins. | Frithz-Lindsten et al., 1998; Dasgupta et al., 2006 |

¹ For the purpose of this table, *P. aeruginosa* is considered a plant pathogen due to its ability to infect plants. Nevertheless, most of the information summarized here was found using animal systems or strains that infect animals.

Overall hypothesis:

Xylella fastidiosa pathogenicity depends upon xylem sap chemical components. Different minerals present in xylem sap act to regulate bacterial virulence by promoting or inhibiting growth, attachment, and aggregation.

Main objective:

Determine the relationship between *Xylella fastidiosa* infection and calcium (Ca) content of the host and its implications in the initial aspects of colonization, including twitching motility, cell attachment, and biofilm formation.

Specific objectives:

1. Determine the effects of Ca on biofilm formation, cell adhesion, twitching motility, and exopolysaccharide formation in vitro
2. Determine the molecular mechanism of Ca regulation of twitching motility
3. Establish in planta the effect of Ca on the expression of symptoms and disease development

References

- Agrios, G.N. 2005. Plant pathology. Elsevier Academic Press, Amsterdam ; Boston.
- Almeida, R.P.P., Wistrom, C., Hill, B.L., Hashim, J., and Purcell, A.H. 2005. Vector transmission of *Xylella fastidiosa* to dormant grape. Plant Disease 89:419-424.
- Andersen, P.C., Brodbeck, B.V., Oden, S., Shriner, A., and Leite, B. 2007. Influence of xylem fluid chemistry on planktonic growth, biofilm formation and aggregation of *Xylella fastidiosa*. Fems Microbiology Letters 274:210-217.
- Arrizubieta, M.J., Toledo-Arana, A., Amorena, B., Penades, J.R., and Lasa, I. 2004. Calcium inhibits Bap-dependent multicellular behavior in *Staphylococcus aureus*. Journal of Bacteriology 186:7490-7498.
- Barras, F., van Gijsegem, F., and Chatterjee, A.K. 1994. Extracellular enzymes and pathogenesis of soft-rot *Erwinia*. Annual Review of Phytopathology 32:201-234.
- Bazylinski, D.A., and Frankel, R.B. 2004. Magnetosome formation in prokaryotes. Nat Rev Micro 2:217-230.
- Berkelman, T., Garret-Engele, P., and Hoffman, N.E. 1994. The pacL gene of *Synechococcus* sp. strain PCC 7942 encodes a Ca(2+)-transporting ATPase. Journal of Bacteriology 176:4430-4436.
- Bilecen, K., and Yildiz, F.H. 2009. Identification of a calcium-controlled negative regulatory system affecting *Vibrio cholerae* biofilm formation. Environmental Microbiology 11:2015-2029.
- Borkow, G., and Gabbay, J. 2005. Copper as a biocidal tool. Current medicinal chemistry 12:2163-2175.
- Burdman, S., Bahar, O., Parker, J.K., and De La Fuente, L. 2011. Involvement of type IV pili in pathogenicity of plant pathogenic bacteria. Genes 2:706-735.
- Campbell, A.K. 1990. Calcium as an intracellular regulator. Proceedings of the Nutrition Society 49:51-56.
- Case, R.M., Eisner, D., Gurney, A., Jones, O., Muallem, S., and Verkhatsky, A. 2007. Evolution of calcium homeostasis: from birth of the first cell to an omnipresent signalling system. Cell Calcium 42:345-350.
- Caserta, R., Takita, M.A., Targon, M.L., Rosselli-Murai, L.K., de Souza, A.P., Peroni, L., Stach-Machado, D.R., Andrade, A., Labate, C.A., Kitajima, E.W., Machado, M.A., and de Souza, A.A. 2010. Expression of *Xylella fastidiosa* fimbrial and afimbrial proteins during biofilm formation. Appl Environ Microbiol 76:4250-4259.
- Chatterjee, S., Newman, K.L., and Lindow, S.E. 2008a. Cell-to-cell signaling in *Xylella fastidiosa* suppresses movement and xylem vessel colonization in grape. Mol. Plant Microbe Interact. 21:1309-1315.
- Chatterjee, S., Almeida, R.P., and Lindow, S. 2008b. Living in two worlds: the plant and insect lifestyles of *Xylella fastidiosa*. Annu Rev Phytopathol 46:243-271.
- Chauvaux, S., Beguin, P., Aubert, J.P., Bhat, K.M., Gow, L.A., Wood, T.M., and Bairoch, A. 1990. Calcium-binding affinity and calcium-enhanced activity of *Clostridium thermocellum* endoglucanase-D. Biochemical Journal 265:261-265.
- Chenal, A., Guijarro, J.I., Raynal, B., Delepierre, M., and Ladant, D. 2009. RTX calcium binding motifs are intrinsically disordered in the absence of calcium. The Journal of Biological Chemistry 284:1781-1789.

- Choat, B., Gambetta, G.A., Wada, H., Shackel, K.A., and Matthews, M.A. 2009. The effects of Pierce's disease on leaf and petiole hydraulic conductance in *Vitis vinifera* cv. Chardonnay. *Physiologia Plantarum* 136:384-394.
- Conn, S.J., Gilliam, M., Athman, A., Schreiber, A.W., Baumann, U., Moller, I., Cheng, N.H., Stancombe, M.A., Hirschi, K.D., Webb, A.A., Burton, R., Kaiser, B.N., Tyerman, S.D., and Leigh, R.A. 2011. Cell-specific vacuolar calcium storage mediated by CAX1 regulates apoplastic calcium concentration, gas exchange, and plant productivity in *Arabidopsis*. *The Plant Cell* 23:240-257.
- Cruz, L.F., Cobine, P.A., and De La Fuente, L. 2012. Calcium increases *Xylella fastidiosa* surface attachment, biofilm formation, and twitching motility. *Appl Environ Microbiol* 78:1321-1331.
- Cucarella, C., Solano, C., Valle, J., Amorena, B., Lasa, I., and Penades, J.R. 2001. Bap, a *Staphylococcus aureus* surface protein involved in biofilm formation. *Journal of Bacteriology* 183:2888-2896.
- Dasgupta, N., Ashare, A., Hunninghake, G.W., and Yahr, T.L. 2006. Transcriptional induction of the *Pseudomonas aeruginosa* type III secretion system by low Ca^{2+} and host cell contact proceeds through two distinct signaling pathways. *Infection and Immunity* 74:3334-3341.
- Datnoff, L.E., Elmer, W.H., and Huber, D.M. 2007. Mineral nutrition and plant disease. American Phytopathological Society, St. Paul, Minn.
- Davis, M.J., Whitcomb, R.F., and Gillaspie, A.G.J. 1981. Fastidious bacteria of plant vascular tissue and invertebrates (including so called rickettsia-like bacteria). Pages 2172-2188 in: *The prokaryotes : a handbook on habitats, isolation, and identification of bacteria*, M.P. Starr, H. Stolp, H.G. Truper, A. Balows, and H.G. Schlegel, eds. Springer-Verlag, Berlin ; New York.
- De La Fuente, L., Burr, T.J., and Hoch, H.C. 2007. Mutations in type I and type IV pilus biosynthetic genes affect twitching motility rates in *Xylella fastidiosa*. *J Bacteriol* 189:7507-7510.
- De Souza, A.A., Takita, M.A., Pereira, E.O., Coletta-Filho, H.D., and A., M.M. 2005. Expression of pathogenicity-related genes of *Xylella fastidiosa* *in vitro* and in planta. *Current Microbiology* 50:223-228.
- Deng, W., Li, Y., Hardwidge, P.R., Frey, E.A., Pfuetzner, R.A., Lee, S., Gruenheid, S., Strynacka, N.C.J., Puente, J.L., and Finlay, B.B. 2005. Regulation of type III secretion hierarchy of translocators and effectors in attaching and effacing bacterial pathogens. *Infection and Immunity* 73:2135-2146.
- Desrosiers, M.G., Gambel, A.M., and Menick, D.R. 1993. Purification of a prokaryotic P-type calcium ATPase. *Biophysical Journal* 64:A335-A335.
- Dominguez, D.C. 2004. Calcium signaling in bacteria. *Mol Microbiol* 54:291-297.
- Durell, S.R., and Guy, H.R. 2001. A putative prokaryote voltage-gated Ca^{2+} channel with only one 6TM motif per subunit. *Biochem Biophys Res Commun* 281:741 - 746.
- Espinosa-Urgel, M., Salido, A., and Ramos, J.L. 2000. Genetic analysis of functions involved in adhesion of *Pseudomonas putida* to seeds. *Journal of Bacteriology* 182:2363-2369.
- Fernandez-Morales, J.C., Padin, J.F., Maroto, M., Calvo, E., Vestring, S., Garcia, A.G., and de Diego, A.M.G. 2011. Blockers of T-Type Ca^{2+} Channels Also Inhibit High Voltage Activated Calcium Currents. *Basic & Clinical Pharmacology & Toxicology* 109:54-54.

- Flego, D., Pirhonen, M., Saarilahti, H., Palva, T.K., and Palva, E.T. 1997. Control of virulence gene expression by plant calcium in the phytopathogen *Erwinia carotovora*. *Molecular Microbiology* 25:831-838.
- Fletcher, M. 1988. Attachment of *Pseudomonas fluorescens* to glass and influence of electrolytes on bacterium substratum separation distance. *Journal of Bacteriology* 170:2027-2030.
- Francis, M.S., Lloyd, S.A., and Wolf-Watz, H. 2001. The type III secretion chaperone LcrH cooperates with YopD to establish a negative, regulatory loop for control of Yop synthesis in *Yersinia pseudotuberculosis*. *Molecular Microbiology* 42:1075-1093.
- Frithz-Lindsten, E., Holmström, A., Jacobsson, L., Soltani, M., Olsson, J., Rosqvist, R., and Forsberg, Å. 1998. Functional conservation of the effector protein translocators PopB/YopB and PopD/YopD of *Pseudomonas aeruginosa* and *Yersinia pseudotuberculosis*. *Molecular Microbiology* 29:1155-1165.
- Galan, J.E., and Collmer, A. 1999. Type III secretion machines: bacterial devices for protein delivery into host cells. *Science* 284:1322-1328.
- Galan, J.E., and Wolf-Watz, H. 2006. Protein delivery into eukaryotic cells by type III secretion machines. *Nature* 444:567-573.
- Gambel, A.M., Desrosiers, M.G., and Menick, D.R. 1992. Calcium transport and ATP hydrolysis by a novel calcium ATPase of *Flavobacterium odoratum*. *Faseb Journal* 6:A120-A120.
- Gambetta, G.A., Fei, J., Rost, T.L., and Matthews, M.A. 2007. Leaf scorch symptoms are not correlated with bacterial populations during Pierce's disease. *J. Exp. Bot.* 58:4037-4046.
- Gangola, P., and Rosen, B.P. 1987. Maintenance of intracellular calcium in *Escherichia coli*. *Journal of Biological Chemistry* 262:12570-12574.
- Gifford, J.L., Walsh, M.P., and Vogel, H.J. 2007. Structures and metal-ion-binding properties of the Ca²⁺-binding helix-loop-helix EF-hand motifs. *Biochemical Journal* 405:199-221.
- Guilhabert, M.R., and Kirkpatrick, B.C. 2005. Identification of *Xylella fastidiosa* antivirulence genes: hemagglutinin adhesins contribute to *Xylella fastidiosa* biofilm maturation and colonization and attenuate virulence. *Molecular Plant-Microbe Interactions* 18:856-868.
- Hakansson, S., Bergman, T., Vanooteghem, J.C., Cornelis, G., and Wolfwatz, H. 1993. Yopb and Yopd constitute a novel class of *Yersinia* Yop proteins. *Infection and Immunity* 61:71-80.
- Hall-Stoodley, L., Costerton, J.W., and Stoodley, P. 2004. Bacterial biofilms: from the natural environment to infectious diseases. *Nature Reviews Microbiology* 2:95-108.
- He, S.Y. 1998. Type III protein secretion systems in plant and animal pathogenic bacteria. *Annual Review of Phytopathology* 36:363-392.
- Hepler, P.K. 2005. Calcium: a central regulator of plant growth and development. *The Plant Cell Online* 17:2142-2155.
- Hinsa, S.M., Espinosa-Urgel, M., Ramos, J.L., and O'Toole, G.A. 2003. Transition from reversible to irreversible attachment during biofilm formation by *Pseudomonas fluorescens* WCS365 requires an ABC transporter and a large secreted protein. *Molecular Microbiology* 49:905-918.
- Hofer, A.M., and Lefkimiatis, K. 2007. Extracellular calcium and cAMP: second messengers as "third messengers"? *Physiology* 22:320-327.
- Hopkins, D.L. 1989. *Xylella fastidiosa* - xylem-limited bacterial pathogen of plants. *Annual Review of Phytopathology* 27:271-290.
- Hopkins, D.L., and Purcell, A.H. 2002. *Xylella fastidiosa*: cause of Pierce's disease of grapevine and other emergent diseases. *Plant Disease* 86:1056-1066.

- Ivey, D.M., Guffanti, A.A., Zemsky, J., Pinner, E., Karpel, R., Padan, E., Schuldiner, S., and Krulwich, T.A. 1993. Cloning and characterization of a putative $\text{Ca}^{2+}/\text{H}^{+}$ antiporter gene from *Escherichia coli* upon functional complementation of $\text{Na}^{+}/\text{H}^{+}$ antiporter-deficient strains by the overexpressed gene. *Journal of Biological Chemistry* 268:11296-11303.
- Jaiswal, J.K. 2001. Calcium - how and why? *Journal of Biosciences* 26:357-363.
- Kanamaru, K., Kashiwagi, S., and Mizuno, T. 1993. The cyanobacterium, *Synechococcus* sp. PCC7942, possesses two distinct genes encoding cation-transporting P-type ATPases. *FEBS Letters* 330:99-104.
- Koishi, R., Xu, H., Ren, D., Navarro, B., Spiller, B.W., Shi, Q., and Clapham, D.E. 2004. A superfamily of voltage-gated sodium channels in bacteria. *The Journal of Biological Chemistry* 279:9532-9538.
- Kundla, J., Batistic, O., and Hashimoto, K. 2010. Calcium signals: the lead currency of plant information processing. *The Plant Cell* 22 March 2010.
- Lefèvre, C.T., Santini, C.-L., Bernadac, A., Zhang, W.-J., Li, Y., and Wu, L.-F. 2010. Calcium ion-mediated assembly and function of glycosylated flagellar sheath of marine magnetotactic bacterium. *Molecular Microbiology* 78:1304-1312.
- Leite, B., Ishida, M.L., Alves, E., Carrer, H., Pascholati, S.F., and Kitajima, E.W. 2002. 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* 35:645-650.
- Li, Y., Hao, G., Galvani, C.D., Meng, Y., De La Fuente, L., Hoch, H.C., and Burr, T.J. 2007. Type I and type IV pili of *Xylella fastidiosa* affect twitching motility, biofilm formation and cell-cell aggregation. *Microbiology* 153:719-726.
- Linhartová, I., Bumba, L., Mašín, J., Basler, M., Osička, R., Kamanová, J., Procházková, K., Adkins, I., Hejnová-Holubová, J., Sadílková, L., Morová, J., and Šebo, P. 2010. RTX proteins: a highly diverse family secreted by a common mechanism. *FEMS Microbiology Reviews* 34:1076-1112.
- Lodish, H.F. (2000). *Molecular cell biology* (New York: W.H. Freeman).
- Marschner, H. 1995. *Mineral nutrition of higher plants*. Academic Press, San Diego, California.
- Martins, A., Machado, L., Costa, S., Cerca, P., Spengler, G., Viveiros, M., and Amaral, L. 2011. Role of calcium in the efflux system of *Escherichia coli*. *International Journal of Antimicrobial Agents* 37:410-414.
- Matsushita, T., Hirata, H., and Kusaka, I. 1989. Calcium channels in bacteria - purification and characterization. *Annals of the New York Academy of Sciences* 560:426-429.
- Meng, Y.Z., Li, Y.X., Galvani, C.D., Hao, G.X., Turner, J.N., Burr, T.J., and Hoch, H.C. 2005. Upstream migration of *Xylella fastidiosa* via pilus-driven twitching motility. *Journal of Bacteriology* 187:5560-5567.
- Michiels, J., Xi, C.W., Verhaert, J., and Vanderleyden, J. 2002. The functions of Ca^{2+} in bacteria: a role for EF-hand proteins? *Trends in Microbiology* 10:87-93.
- Mila, I., Scalbert, A., and Expert, D. 1996. Iron withholding by plant polyphenols and resistance to pathogens and rots. *Phytochemistry* 42:1551-1555.
- Moens, S., and Vanderleyden, J. 1996. Functions of bacterial flagella. *Critical Reviews in Microbiology* 22:67-100.
- Muller, S., Feldman, M.F., and Cornelis, G.R. 2001. The Type III secretion system of Gram-negative bacteria: a potential therapeutic target? *Expert Opinion on Therapeutic Targets* 5:327-339.

- Nardini, M., Lang, D.A., Liebeton, K., Jaeger, K.-E., and Dijkstra, B.W. 2000. Crystal structure of *Pseudomonas aeruginosa* lipase in the open conformation: the prototype for family I.1 of bacterial lipases. The Journal of Biological Chemistry: M003903200.
- Newman, K.L., R. P. P. Almeida, A. H. Purcell, and S. E. Lindow. 2003. Use of a green fluorescent strain for analysis of *Xyella fastidiosa* colonization of *Vitis vinifera*. Appl Environ Microbiol 69:7319-7327.
- Norris, V., Grant, S., Freestone, P., Canvin, J., Sheikh, F.N., Toth, I., Trinei, M., Modha, K., and Norman, R.I. 1996. Calcium signalling in bacteria. J Bacteriol 178:3677-3682.
- Ohyama, T., Igarashi, K., and Kobayashi, H. 1994. Physiological role of the *chaA* gene in sodium and calcium circulations at a high pH in *Escherichia coli*. Journal of Bacteriology 176:4311-4315.
- Onoda, T., Enokizono, J., Kaya, H., Oshima, A., Freestone, P., and Norris, V. 2000. Effects of calcium and calcium chelators on growth and morphology of *Escherichia coli* L-form NC-7. J Bacteriol 182:1419-1422.
- Orans, J., Johnson, M.D.L., Coggan, K.A., Sperlazza, J.R., Heiniger, R.W., Wolfgang, M.C., and Redinbo, M.R. 2009. Crystal structure analysis reveals *Pseudomonas* PilY1 as an essential calcium-dependent regulator of bacterial surface motility. Proceedings of the National Academy of Sciences of the United States of America.
- Ordal, G.W. 1977. Calcium ion regulates chemotactic behaviour in bacteria. Nature 270:66-67.
- Ottemann, K.M., and Miller, J.F. 1997. Roles for motility in bacterial–host interactions. Molecular Microbiology 24:1109-1117.
- Pacheco, F.T.H., Silva-Stenico, M.E., Etchegaray, A., Gomes, J.E., Carrilho, E., and Tsai, S.M. 2006. Specific amplification of iron receptor genes in *Xylella fastidiosa* strains from different hosts. Genetics and molecular biology 29:137-141.
- Pagel, W., and Heitefuss, R. 1990. Enzyme activities in soft rot pathogenesis of potato tubers: effects of calcium, pH, and degree of pectin esterification on the activities of polygalacturonase and pectate lyase. Physiological and Molecular Plant Pathology 37:9-25.
- Patrauchan, M.A., Sarkisova, S.A., and Franklin, M.J. 2007. Strain-specific proteome responses of *Pseudomonas aeruginosa* to biofilm-associated growth and to calcium. Microbiology 153:3838-3851.
- Patrauchan, M.A., Sarkisova, S., Sauer, K., and Franklin, M.J. 2005. Calcium influences cellular and extracellular product formation during biofilm-associated growth of a marine *Pseudoalteromonas* sp. Microbiology-Sgm 151:2885-2897.
- Paulsen, I.T., Nguyen, L., Sliwinski, M.K., Rabus, R., and Saier, M.H. 2000. Microbial genome analyses: comparative transport capabilities in eighteen prokaryotes. Journal of Molecular Biology 301:75-100.
- Pérez-Donoso, A.G., Sun, Q., Roper, M.C., Greve, L.C., Kirkpatrick, B., and Labavitch, J.M. 2010. Cell wall-degrading enzymes enlarge the pore size of intervessel pit membranes in healthy and *Xylella fastidiosa*-infected grapevines. Plant Physiology 152:1748-1759.
- Portnoy, D.A., Moseley, S.L., and Falkow, S. 1981. Characterization of plasmids and plasmid-associated determinants of *Yersinia enterocolitica* pathogenesis. Infection and Immunity 31:775-782.
- Raeymaekers, L., Wuytack, E.Y., Willems, I., Michiels, C.W., and Wuytack, F. 2002. Expression of a P-type Ca²⁺-transport ATPase in *Bacillus subtilis* during sporulation. Cell Calcium 32:93-103.

- Reed, W.P., and Williams Jr, R.C. 1978. Bacterial adherence: First step in pathogenesis of certain infections. *Journal of Chronic Diseases* 31:67-72.
- Reusch, R.N., Huang, R., and Bramble, L.L. 1995. Poly-3-hydroxybutyrate/polyphosphate complexes form voltage-activated Ca^{2+} channels in the plasma membranes of *Escherichia coli*. *Biophys J* 69:754-766.
- Rimpilainen, M., Forsberg, A., and Wolfwatz, H. 1992. A novel protein, Lcrq, involved in the low-calcium response of *Yersinia pseudotuberculosis* shows extensive homology to YopH. *Journal of Bacteriology* 174:3355-3363.
- Robinson, J.B., Tuovinen, O.H., and Bauer, W.D. 1992. Role of divalent cations in the subunit associations of complex flagella from *Rhizobium meliloti*. *Journal of Bacteriology* 174:3896-3902.
- Rodrigues, C., Takita, M., Coletta-Filho, H., Olivato, J., Caserta, R., Machado, M., and Souza, A. 2008. Copper resistance of biofilm cells of the plant pathogen *Xylella fastidiosa*. *Applied Microbiology and Biotechnology* 77:1145-1157.
- Rodríguez-Navarro, D.N., Dardanelli, M.S., and Ruíz-Saínz, J.E. 2007. Attachment of bacteria to the roots of higher plants. *FEMS Microbiology Letters* 272:127-136.
- Roper, M., Greve, L., Labavitch, J., and Kirkpatrick, B. 2005. Polygalacturonase is required for *Xylella fastidiosa* colonization and pathogenicity in *Vitis vinifera* cv. Chardonnay grapevines. *Phytopathology* 95:S90-S90.
- Rosch, J.W., Sublett, J., Gao, G., Wang, Y.D., and Tuomanen, E.I. 2008. Calcium efflux is essential for bacterial survival in the eukaryotic host. *Molecular Microbiology* 70:435-444.
- Rose, R.K. 2000. The role of calcium in oral streptococcal aggregation and the implications for biofilm formation and retention. *Biochimica Et Biophysica Acta-General Subjects* 1475:76-82.
- Rouch, D.A., and Brown, N.L. 1997. Copper-inducible transcriptional regulation at two promoters in the *Escherichia coli* copper resistance determinant *pco*. *Microbiology* 143:1191-1202.
- Sarkisova, S., Patrauchan, M.A., Berglund, D., Nivens, D.E., and Franklin, M.J. 2005. Calcium-induced virulence factors associated with the extracellular matrix of mucoid *Pseudomonas aeruginosa* biofilms. *Journal of Bacteriology* 187:4327-4337.
- Schaad, N.W., Postnikova, E., Lacy, G., Fatmi, M.B., and Chang, C.J. 2004. *Xylella fastidiosa* subspecies: *X. fastidiosa* subsp *piercei*, subsp nov., *X. fastidiosa* subsp *multiplex* subsp. nov., and *X. fastidiosa* subsp *pauca* subsp nov. *Syst. Appl. Microbiol.* 27:290-300.
- Schuenzel, E.L., Scally, M., Stouthamer, R., and Nunney, L. 2005. A multigene phylogenetic study of clonal diversity and divergence in North American strains of the plant pathogen *Xylella fastidiosa*. *Appl Environ Microbiol* 71:3832-3839.
- Shemarova, I.V., and Nesterov, V.P. 2005. Evolution of mechanisms of Ca^{2+} -signaling: role of calcium ions in signal transduction in prokaryotes. *Journal of Evolutionary Biochemistry and Physiology* 41:12-19.
- Silva-Stenico, M., Pacheco, F., Pereira-Filho, E., Rodrigues, J., Souza, A., Etchegaray, A., Gomes, J., and Tsai, S. 2009. Nutritional deficiency in citrus with symptoms of citrus variegated chlorosis disease. *Brazilian Journal of Biology* 69:859-864.
- Silva-Stenico, M.E., Pacheco, F.T., Rodrigues, J.L., Carrilho, E., and Tsai, S.M. 2005. Growth and siderophore production of *Xylella fastidiosa* under iron-limited conditions. *Microbiological research* 160:429-436.

- Slonczewski, J., Foster, J.W., and Gillen, K.M. 2009. Microbiology: an evolving science. W.W. Norton & Co., New York.
- Smit, G., Logman, T.J., Boerrigter, M.E., Kijne, J.W., and Lugtenberg, B.J. 1989. Purification and partial characterization of the *Rhizobium leguminosarum* biovar *viciae* Ca²⁺-dependent adhesin, which mediates the first step in attachment of cells of the family Rhizobiaceae to plant root hair tips. *Journal of Bacteriology* 171:4054-4062.
- Smith, C.A., Toogood, H.S., Baker, H.M., Daniel, R.M., and Baker, E.N. 1999. Calcium-mediated thermostability in the subtilisin superfamily: the crystal structure of *Bacillus* Ak.1 protease at 1.8 Å resolution. *Journal of Molecular Biology* 294:1027-1040.
- Smith, R.J. 1995. Calcium and bacteria. *Adv Microb Physiol* 37:83-133.
- Snyder, L., and Champness, W. 2003. Molecular genetics of bacteria. ASM Press, Washington, D.C.
- Stevenson, J.F., Matthews, M.A., and Ros, T.L. 2004. Grapevine susceptibility to Pierce's disease I: relevance to hydraulic architecture. *American Journal of Enology and Viticulture* 55:228-237.
- Stevenson, J.F., Matthews, M.A., and Rost, T.L. 2005. The developmental anatomy of Pierce's disease symptoms in grapevines: green islands and matchsticks. *Plant Disease* 89:543-548.
- Straley, S.C., Plano, G.V., Skrzypek, E., Haddix, P.L., and Fields, K.A. 1993. Regulation by Ca²⁺ in the *Yersinia* low-Ca²⁺ response. *Molecular Microbiology* 8:1005-1010.
- Swart, S., Lugtenberg, B.J.J., Smit, G., and Kijne, J.W. 1994. Rhicadhesin-mediated attachment and virulence of an *Agrobacterium tumefaciens* Chvb mutant can be restored by growth in a highly osmotic medium. *Journal of Bacteriology* 176:3816-3819.
- Thorne, E.T., Stevenson, J.F., Rost, T.L., Labavitch, J.M., and Matthews, M.A. 2006. Pierce's disease symptoms: comparison with symptoms of water deficit and the impact of water deficits. *American Journal of Enology and Viticulture* 57:1-11.
- Tisa, L.S., and Adler, J. 1992. Calcium ions are involved in *Escherichia coli* chemotaxis. *Proceedings of the National Academy of Sciences of the United States of America* 89:11804-11808.
- Tisa, L.S., and Adler, J. 1995. Cytoplasmic free-Ca²⁺ level rises with repellents and falls with attractants in *Escherichia coli* chemotaxis. *Proceedings of the National Academy of Sciences of the United States of America* 92:10777-10781.
- Torrecilla, I., Leganés, F., Bonilla, I., and Fernández-Piñas, F. 2000. Use of recombinant aequorin to study calcium homeostasis and monitor calcium transients in response to heat and cold shock in cyanobacteria. *Plant Physiology* 123:161-176.
- Torres, A.G., Perna, N.T., Burland, V., Ruknudin, A., Blattner, F.R., and Kaper, J.B. 2002. Characterization of Cah, a calcium-binding and heat-extractable autotransporter protein of enterohaemorrhagic *Escherichia coli*. *Molecular Microbiology* 45:951-966.
- Trombe, M.C. 1999. Calcium signaling in *Streptococcus pneumoniae*: implication of the kinetics of calcium transport. *Microbial Drug Resistance-Mechanisms Epidemiology and Disease* 5:247-252.
- Trombe, M.C., Rieux, V., and Baille, F. 1994. Mutations which alter the kinetics of calcium-transport alter the regulation of competence in *Streptococcus pneumoniae*. *Journal of Bacteriology* 176:1992-1996.
- Van Petegem, F., and Minor, D.L., Jr. 2006. The structural biology of voltage-gated calcium channel function and regulation. *Biochem Soc Trans* 34:887-893.

- Waditee, R., Hossain, G.S., Tanaka, Y., Nakamura, T., Shikata, M., Takano, J., Takabe, T., and Takabe, T. 2004. Isolation and functional characterization of Ca²⁺/H⁺ antiporters from cyanobacteria. *Journal of Biological Chemistry* 279:4330-4338.
- Waldron, K.J., and Robinson, N.J. 2009. How do bacterial cells ensure that metalloproteins get the correct metal? *Nature reviews. Microbiology* 7:25-35.
- Watkins, N.J., Knight, M.R., Trewavas, A.J., and Campbell, A.K. 1995. Free calcium transients in chemotactic and non-chemotactic strains of *Escherichia coli* determined by using recombinant aequorin. *Biochemical Journal* 306:865-869.
- Wells, J.M., Raju, B.C., Hung, H.Y., Weisburg, W.G., Mandelcopaul, L., and Brenner, D.J. 1987. *Xylella fastidiosa* gen. nov., sp. nov: Gram-negative, xylem-limited, fastidious plant bacteria related to *Xanthomonas* spp. *Int J Syst Bacteriol* 37:136-143.
- Wilson, J.W., Schurr, M.J., LeBlanc, C.L., Ramamurthy, R., Buchanan, K.L., and Nickerson, C.A. 2002. Mechanisms of bacterial pathogenicity. *Postgraduate Medical Journal* 78:216-224.
- Yildiz, F.H., Dolganov, N.A., and Schoolnik, G.K. 2001. VpsR, a member of the response regulators of the two-component regulatory systems, is required for expression of vps biosynthesis genes and EPSETr-associated phenotypes in *Vibrio cholerae* O1 El Tor. *Journal of Bacteriology* 183:1716-1726.
- Yu, X.-C., and Margolin, W. 1997. Ca²⁺-mediated GTP-dependent dynamic assembly of bacterial cell division protein FtsZ into asters and polymer networks in vitro. *EMBO J* 16:5455-5463.
- Zaini, P.A., Fogaca, A.C., Lupo, F.G.N., Nakaya, H.I., Vencio, R.Z.N., and da Silva, A.M. 2008. The iron stimulon of *Xylella fastidiosa* includes genes for type IV pilus and colicin V-like bacteriocins. *Journal of Bacteriology* 190:2368-2378.
- Zhou, Y., Yang, W., Kirberger, M., Lee, H.W., Ayalasomayajula, G., and Yang, J.J. 2006. Prediction of EF-hand calcium-binding proteins and analysis of bacterial EF-hand proteins. *Proteins* 65:643-655.

Chapter 2 Calcium increases surface attachment, biofilm formation, and twitching motility in *Xylella fastidiosa*

Abstract

Xylella fastidiosa is a plant pathogenic bacterium that forms biofilms inside xylem vessels; therefore the chemical composition of the xylem sap influences bacterial infection. In this work the effect of calcium on the production of *X. fastidiosa* biofilm and movement was analyzed under *in vitro* conditions. After a dose-response study in 96-well plates with eight metals, the strongest effect on biofilm formation was observed when supplementing media with at least 2mM CaCl₂. Removal of Ca by extracellular (ethylene glycol tetraacetic acid, EGTA 1.5mM) and intracellular (1,2-bis (o-aminophenoxy) ethane-N,N',N',N'-tetraacetic acid acetoxymethyl ester, BAPTA/AM 75 μM) chelators reduced biofilm formation without compromising planktonic growth. Experiments using microfluidic chambers showed that concentration of Ca influences adhesion force to substrate, biofilm thickness, cell-to-cell aggregation, and twitching motility. The effect of Ca in attachment to substrates was lost when cells were treated with tetracycline, suggesting that Ca has a metabolic or regulatory role in cell adhesion. A double mutant (*fimA*⁻/*pilO*⁻) lacking type I and type IV pili did not improve biofilm formation or attachment when Ca was added to the media, while single mutants in type I (*fimA*⁻) or type IV (*pilB*⁻) pili formed more biofilm under higher Ca concentrations. Media Ca concentration did not significantly influence the levels of exopolysaccharide produced. Our findings indicate that the role of Ca in biofilm formation may be related to the initial surface and cell-to-cell attachment

and colonization stages of biofilm establishment, which rely on critical functions by fimbrial structures.

Introduction

The xylem-limited gram negative bacterium *Xylella fastidiosa* is the causal agent of many economically important plant diseases including Pierce's Disease (PD) of grapevines, citrus-variegated chlorosis (CVC) (Hopkins and Purcell, 2002), and the emerging bacterial leaf scorch in blueberries (Chang et al., 2009). Other crops including alfalfa, peach, plum, almond, and coffee and many forest trees and landscape plants including oak, elm, maple, and oleander can also be infected by this bacterium. Characteristic disease symptoms, such as leaf scorch, have been associated with the blockage of xylem vessels by bacterial biofilms, which may result in water deficit (Stevenson et al., 2004). Even though the exact mechanism of *X. fastidiosa* pathogenesis is still unclear, disease development depends on the ability of the bacterium to multiply and become systemic in the host (Chatterjee et al., 2008).

Cell attachment and biofilm formation are determinants for host infection by pathogenic bacteria (Romantschuk, 1992). Genes in *X. fastidiosa* encode various surface-associated proteins that are thought to be important for attachment, intra-plant migration, host colonization, and biofilm formation. Proteins involved in these processes include fimbrial adhesins, which are components of type I and type IV pili that are located at the same cell pole (Meng et al., 2005). The type I pilus is responsible for cell anchorage to surfaces (De la Fuente et al., 2007b), while type IV pilus extends and retracts to assist in cell twitching motility (Meng et al., 2005). Afimbrial adhesin proteins have also been implicated in bacterial cell-to-cell attachment and biofilm maturation (Guilhabert and Kirkpatrick, 2005).

Bacterial biofilms provide protection to the community of cells from antimicrobial compounds and dehydration, promote cell-to-cell signaling interactions, and help to optimize nutrient uptake (Hall-Stoodley et al., 2004). Inside the host environment, factors such as the availability of water and nutrients can limit the formation of biofilm (Ramey et al., 2004). Different environments vary in the concentrations of solutes; therefore, bacteria have evolved mechanisms for regulating cellular nutrient concentrations (Snyder and Champness, 2003). For instance, bacterial biofilms have been thought to improve nutrient acquisition through formation of exopolysaccharides (EPS), which have high capacity for ion retention, a strategy particularly important in nutrient dilute conditions such as in xylem sap (Costerton et al., 1995). In *X. fastidiosa* the role of biofilm formation for bacterial infection has been demonstrated (Newman, 2003; Chatterjee et al., 2008); however, the specific host factors determining the dynamics of biofilm formation are still under investigation.

X. fastidiosa survives in the xylem, which is the conduit for distribution of nutrients from the root to the leaves, including micro- and macronutrients required for multiple functions within prokaryotic and eukaryotic cells (Hansch and Mendel, 2009; Maathuis, 2009). These essential minerals are under tight homeostatic control because, although required, they are also toxic at high concentrations. Availability of minerals and trace elements affects host-pathogen interactions, especially pathogen survival, expression of virulence traits, and host physiology. Calcium levels modulate biofilm structure in *Vibrio cholerae* (Kierek and Watnick, 2003), expression of Type III secretion apparatus and effector proteins in *Yersinia pestis* (DeBord et al., 2003) and *Pseudomonas aeruginosa* (Dasgupta et al., 2006), and production of hydrolytic

enzymes (Polygalacturonase and pectate lyase) in *Pectobacterium carotovora* (Flego et al., 1997), all of them considered virulence determinants. In addition, in animal and plant hosts, calcium modulates defense responses, based on regulatory systems that rely on this metal as a secondary messenger (Nhieu et al., 2004; Lecourieux et al., 2006).

Calcium, magnesium, and iron have all been implicated in *X. fastidiosa* infection process. However, it has been suggested to be non-specific in some cases (e.g. Ca, Mg) with these elements playing a role as bridges for the adhesion between negatively-charged bacterial cells and xylem vessels (Leite et al., 2002). While previous studies using gene expression showed that Fe regulates virulence factors such as type IV pilus and bacteriocins (Zaini et al., 2008), other studies showed that cells in biofilms were more resistant to Cu than planktonic cells (Rodrigues et al., 2008) and that Cu and Zn present in xylem fluid are correlated to *in vitro* growth of *X. fastidiosa* (Andersen et al., 2007). Moreover, a Zn-protease is induced in *X. fastidiosa* in citrus to utilize free amino acids in the xylem as nitrogen and carbon sources (Purcino et al., 2007). These studies suggested that modulation of the mineral content of the xylem where *X. fastidiosa* is colonizing the host plant will have an impact on the virulence of this bacterium.

In the present work, we studied the effects of concentration of calcium on *X. fastidiosa* biofilm formation and twitching movement *in vitro*. Different *X. fastidiosa* traits implicated in the formation of biofilm, including cell attachment to surfaces, cell-to-cell aggregation, twitching motility, and production of EPS, were analyzed. The presence of Ca enhances biofilm formation, cell attachment, and motility under *in vitro* conditions. No evidence for Ca effect on EPS

production was found. These results suggest that the role of Ca is related to the initial steps in the formation of biofilm.

Materials and methods

Bacterial strains and culture conditions.

Xylella fastidiosa strain Temecula (ATCC 700964) was used as the wild-type (WT) strain in this study. Four mutant strains previously obtained by random mutagenesis using an EZ::TN Tn5 transposon system were also used: *fimA* (PD_0062) null cells, lacking type I pili (Meng et al., 2005); *pilB* (PD_1927) null cells, lacking type IV pili (Meng et al., 2005); and *fimA/pilO* (PD_0062/PD_1693) a double mutant, lacking type I and type IV pili (Li et al., 2007). For confocal microscope studies, a green, fluorescent, protein-expressing mutant of the WT (strain KLN59.3) (Newman, 2003) was used. All *X. fastidiosa* strains were grown on PW (Davis et al., 1980) agar solid medium or PD2 (Davis et al., 1981) liquid medium at 28°C. Stocks of *X. fastidiosa* cultures were stored in PW broth plus 20% glycerol at -80°C.

Biofilm quantification in 96-well plates.

Biofilm formation by *X. fastidiosa* WT and mutant strains was assessed according to Zaini et al. (2009) with some modifications. Briefly, cells cultured for 7 days on PW plates were scraped from the plates and suspended in PD2 media ($OD_{600} = 0.8$). Sterile polystyrene 96-well plates containing 200 µl PD2 per well were inoculated with 10 µl cell suspension. After 5 days of

incubation at 28°C, the total number of cells, planktonic growth (cells in suspension), and biofilm growth (cells adhered to the substrate) was estimated by measuring OD₆₀₀. Planktonic growth was quantified by transferring the broth containing cells in suspension to a new plate. Then, the original 96-well plate was rinsed 3 times with Milli-Q water and stained with 0.1 % crystal-violet to quantify biofilm growth (Zaini et al., 2009).

Supplementation of media with metals and chelators.

Biofilm formation was assessed as described above in cells grown in PD2 supplemented with metals and chelators at different concentrations. PD2 was supplemented with CaCl₂, CoCl₂, CuSO₄, FeSO₄, KCl, MgSO₄, Mn₂(SO₄)₃, or ZnCl₂ in a range of concentrations from 0.1 mM to 2.5 mM. Additionally, biofilm formation was evaluated in the presence of either the extracellular calcium chelator ethylene glycol tetraacetic acid (EGTA), in a range of concentrations from 0.063 mM to 1.5 mM, or the cell-permeant Ca chelator 1,2-bis (o-aminophenoxy) ethane-N,N,N',N'-tetraacetic acid acetoxymethyl ester (BAPTA/AM), in a range of concentrations from 4.2 μM to 100 μM. For all experiments, each plate included a control row (n = 12) of cells grown in non-supplemented PD2. All experiments were replicated at least three times.

Metal quantification.

X. fastidiosa or PD2 media was analyzed by Inductively Coupled Plasma with Optical Emission Spectroscopy (ICP-OES, Perkin Elmer 7100 DV, Waltham, MA) with simultaneous measurement of B, Ca, Co, Cu, Fe, K, Mg, Mn, Mo, Ni, P, S, and Zn. Cells were collected by

centrifugation and washed three times in ultra-pure, metal-free water and once in 0.5 mM EDTA. Equal numbers of cells were acid-digested for 1 hour in sealed tubes at 95°C in 150 µL of metal-free concentrated nitric acid (Optima, Fisher Scientific) as previously described (Yang et al., 2006). As controls, blanks of nitric acid were digested in parallel. Digested samples were centrifuged and supernatants diluted using ultra-pure, metal-free water. Metal concentrations were determined by comparing intensities to a standard curve created from certified metal standards (SPEX, Metuchen, NJ). Available Ca was measured using the fluorescent probes Fluo-5 and Fluo-5AM (Invitrogen, Carlsbad, CA). Standard solutions of 5 mM were prepared in DMSO then diluted into aqueous solution for use. Relative fluorescence (Rf) was measured on a LS50B fluorescence spectrometer (Perkin Elmer, Waltham, MA). Excitation was set at 485 nm (slit width= 3 nm) and emission scanned from 500-600 nm with Rf read at maximum intensity of 520 nm (slit width= 3 nm).

Bacterial cell-to-cell aggregation assessment.

Bacterial aggregation was examined in biofilm cells formed at the air-liquid interface of glass culture tubes containing 5 ml of *X. fastidiosa* WT PD2 liquid cultures incubated with shaking at 28°C for 7 days. Cells were harvested and suspended in 200 µl of Milli-Q water to an equal starting OD₆₀₀ of 0.8. Cell-to-cell aggregation was established via “settling rate” using a UV-Vis 2450 spectrophotometer (Shimadzu scientific instruments, Columbia, MD). The cell suspension was homogenized vigorously by pipetting and placed in the spectrophotometer where OD₆₀₀ was continuously measured for 1 minute. The “settling rate” was calculated as the slope of the linear portion of decreasing change in OD₆₀₀ over time.

Strength of bacterial surface attachment in microfluidic chambers.

The strength of cell attachment to surfaces (adhesion force) was evaluated under different chemical treatments using microfluidic chambers as previously described (De la Fuente et al., 2007b; Li et al., 2007). Briefly, for fabrication of microfluidic chambers, photolithography was used to etch a pattern onto a silicon wafer for casting with polydimethylsiloxane (PDMS). Chambers consist of a molded PDMS body sandwiched between a cover glass and a supporting glass microscope slide. The design used in these experiments (De la Fuente et al., 2007b) has two parallel microchannels (80 μm wide x 3.7 cm long x 50 μm deep), which each have two inlets to allow separate entry of media and bacteria and an outlet to allow media to flow out the other end.

For adhesion force assessments, media treatments evaluated were non-supplemented PD2 (control) and PD2 supplemented with 2 mM Ca, 1.5 mM EGTA, 75 μM BAPTA/AM, or 100 μg ml^{-1} tetracycline. Eight-day-old bacterial cultures were scraped from PW plates, suspended in each media treatment, and homogenized by vortexing. The bacterial suspension was introduced into the microfluidic channels ($\sim 22^\circ\text{C}$) through tubing connected to the chamber. Media flow was controlled by an automated syringe pump (Pico Plus; Harvard Apparatus, Holliston, MA). The microfluidic chambers were mounted on a Nikon Eclipse Ti inverted microscope (Nikon, Melville, NY) and observed at 40 X phase-contrast optics to monitor cell attachment. After approximately 2 hours at a media flow speed of $0.25 \mu\text{l min}^{-1}$, when the cells had attached, flow speed was adjusted to $1.0 \mu\text{l min}^{-1}$ for 1 hour to remove non-attached cells. Media flow was then sequentially increased every 1 min by $10 \mu\text{l/min}$ from $1\text{-}120 \mu\text{l/min}$ to gradually remove attached

cells. Time-lapse microscopy images were acquired every 5 seconds during this time with a Nikon DS-Q1 digital camera (Nikon, Melville, NY) controlled by NIS-Elements software (Nikon, Melville, NY) to record detachment of *X. fastidiosa* cells. The number of attached cells remaining in each frame was scored using NIS-elements software. Total cell counts were averaged across the 12 frames collected each minute to obtain the count for each flow rate. Cell adhesion force was calculated according to De La Fuente et al. (2007b). Each treatment was evaluated at least 7 times in independent microfluidic chamber experiments.

Quantification of exopolysaccharides production by biofilm cells.

Production of EPS was examined in biofilm cells formed at the air-liquid interface of glass culture tubes containing 5 ml of *X. fastidiosa* WT liquid cultures in PD2 incubated with shaking at 28°C for 7 days. PD2 broth was supplemented with either 2 mM Ca or 1.5 mM EGTA. Relative quantification of EPS was performed by a phenol-sulfuric acid assay, which determines the total sugar content of the cells as an indication of EPS production (Dubois et al., 1956). Glucose was used for sugar standard curves and non-supplemented PD2 as the control for the treatments. Each treatment was replicated 10 times. For quantification of the number of *X. fastidiosa* biofilm cells in each treatment, quantitative polymerase chain reaction (qPCR) was performed. DNA from 1/3 the total volume of biofilm suspension was extracted using a modified CTAB protocol (Doyle and Doyle, 1987). qPCR was performed using previously-designed *X. fastidiosa*-specific primers and TaqMan probe set (Francis et al., 2006). qPCR reactions (25 µl total volume) consisted of the following: 1X ABsoluteTM Blue QPCR ROX Mix (ABgene-Thermo Fisher Scientific, Surrey, UK), 0.4 µl FAM-BHQ1-labeled probe, 0.2 µM each primer,

and 1 μ l DNA. Samples were amplified on an ABI 7500 Real Time PCR System (Applied Biosystems, Foster City, CA) using the following cycling parameters: 95°C for 1 min followed by 40 cycles of 95°C for 15 s, 60°C for 1 min. All qPCR runs included a 4-point standard curve prepared from 10-fold serial dilutions of DNA extracted from an *X. fastidiosa* cell suspension of known concentration. Cells in the suspension were enumerated by spread-plating serial dilutions on PW agar and counting colony-forming units (CFU) produced after 2-week incubation at 28°C. qPCR amplification efficiencies were comparable to previously described data (Francis et al., 2006) and standard curves had r^2 values > 0.97 .

Biofilm structure assessment by confocal laser scanning microscopy.

Biofilm structure assessments were performed on glass slides prepared as previously described by O'Toole et al, (1999) with a few modifications. Briefly, 20 μ l of a cell suspension ($OD_{600} = 0.8$) of KLN59.3 (*X. fastidiosa* GFP) was inoculated into 50 ml conical polystyrene tubes, each containing a 22 x 22 mm microscope slide submerged in 10 ml of PD2 broth or PD2 supplemented with either 2 mM $CaCl_2$ or 1.5 mM EGTA. Each treatment was replicated three times. The tubes were incubated at 28°C for 15 days, allowing the formation of biofilm on the surface of the slide.

Biofilm was observed with a Nikon Eclipse Ti confocal ,laser-scanning, inverted microscope (Nikon, Melville, NY) using a 60X oil immersion objective with 488 nm excitation wavelengths. The three-dimensional architecture of the biofilm was assessed by Z-stack images with 1 μ m increments, and images were acquired with a CoolSNAP HQ2 camera (Photometrics, Tucson,

AR). Three-dimensional reconstructions of the images and measurement of biofilm thickness were performed with NIS-elements AR software ver. 3.0 (Nikon Melville, NY).

Twitching motility quantification on agar plates and microfluidic chambers.

Twitching motility was assessed on agar plates. WT cells were spotted in quadruplicate onto PW agar plates supplemented with the following treatments: 2 mM Ca, 1.5 mM EGTA, and non-supplemented PW control. After 7 days of incubation at 28°C, the colony peripheral fringe morphology was examined under the microscope, and fringe width was measured ($n = 3$) for each of the 4 colonies using NIS-Elements software. Each treatment was replicated on 3 plates, for a total of 36 measurements per treatment.

Additionally, twitching motility of WT cells was assessed in microfluidic chambers mounted on an inverted microscope as previously described (De La Fuente et al., 2007a). Cells were introduced into the chambers and incubated for 2 hours before time-lapse images were recorded every 30 seconds for 1 hour. Movement of *X. fastidiosa* cells was quantified by tracking their position frame by frame, and cell speed was calculated by measuring the upstream displacement with respect to time.

Statistical analyses.

All experiments were carried out in a randomized complete design (RCD). Data were imported, linearized, and tabulated in SAS 9.2 (SAS Institute Inc.) using GLIMMIX, which combines a

linear regression and analysis of variance models with normal errors, to check the residuals.

Least-squared means were compared at $P \leq 0.05$ level of significance.

Results

Effect of metal supplementation on biofilm formation.

When different metals were added to PD2 media, Ca and Fe produced a strong increase in biofilm formation in 96-well plates assays compared to the non-supplemented control (Fig 1A). A positive correlation between Ca concentration and biofilm formation was observed for the complete range of Ca concentrations tested. At 2.5 mM of additional Ca, the amount of biofilm formed was 500% higher than the control. Unlike for Ca, the strength of the effect of Fe on biofilm formation decreased for concentrations of Fe higher than 1.8 mM, and at 2.5 mM the increase in biofilm formation was just 150% compared with non-supplemented media (Fig 2-1A). In contrast, increases in the concentrations of Cu, Co, Mn, and Zn inhibited the accumulation of biofilm to 50% compared to cells grown in non-supplemented PD2. No significant differences were found in biofilm accumulation for additions of K and Mg (Fig 2-1A). Planktonic growth was considerably less affected by the addition of these metals. Co, K, Mg, and, Mn produced a reduction in planktonic growth of < 20% (Fig 2-1B). None of the metals significantly increased total growth compared to the control (data not shown).

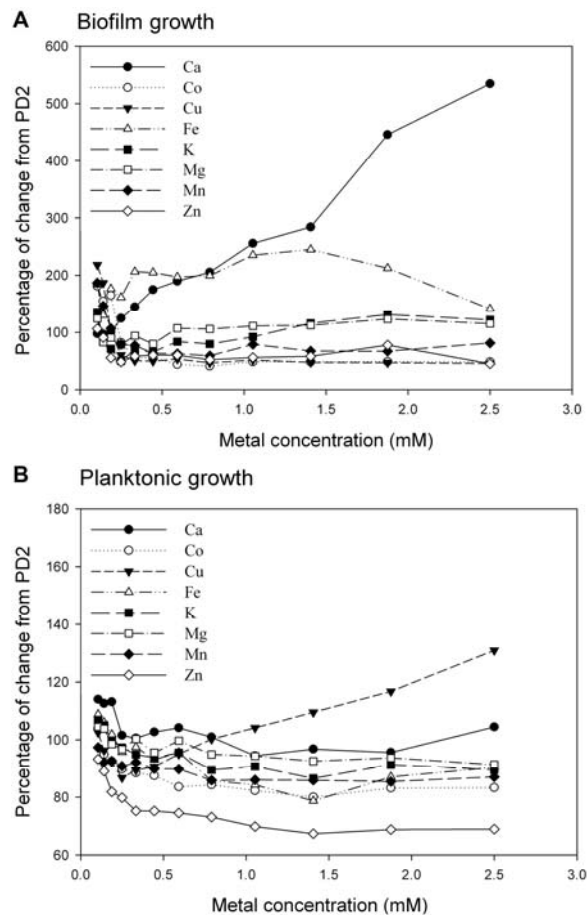


Figure 2-1. Biofilm quantification and planktonic growth of *X. fastidiosa* WT strain in PD2 supplemented with different metals.

Values are expressed relative to the amount of biofilm obtained for *X. fastidiosa* in non-supplemented PD2 (considered 100%). A) biofilm growth, B) planktonic growth. The experiment was repeated three times, and each experiment contained three replicates. Data from a representative experiment are presented.

Effects of calcium chelators on biofilm formation of *X. fastidiosa* WT.

Biofilm formation in 96-well plates in the presence of EGTA and BAPTA/AM, extra- and intracellular calcium chelators, respectively, was quantified. EGTA reduced the formation of biofilm to approximately 40% at all tested concentrations (0.063 mM to 1.5 mM), while the planktonic growth was similar (> 90%) to that found in the cells incubated in control media (Fig 2A).

Similarly, the addition of BAPTA/AM, which is only active as a chelator when the AM ester is cleaved by the intracellular esterases, caused a negative effect on biofilm formation, reducing it to approximately 40% at concentrations 30 μ M or higher (Fig 2B). Under the same conditions, addition of BAPTA/AM reduced planktonic growth to approximately 80% compared to control media.

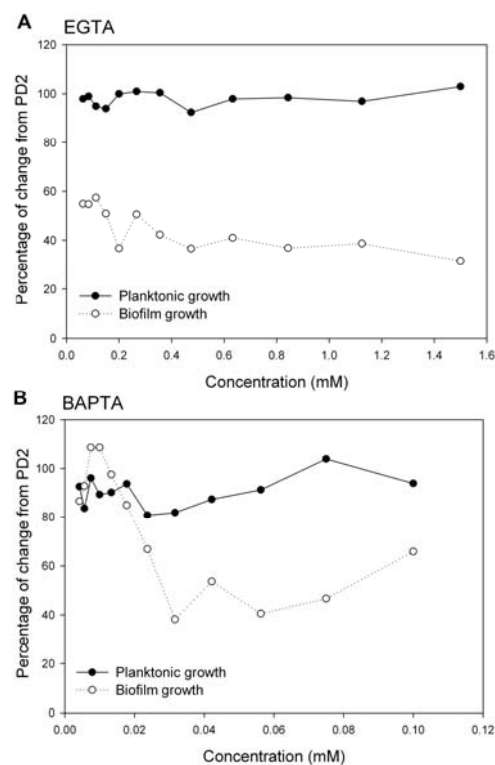


Figure 2-2. Effect of removal of Ca on *X. fastidiosa* WT strain biofilm and planktonic growth.

Ca- extracellular chelator EGTA (A) and intracellular BAPTA/AM (B) were added to the media and biofilm and bacterial growth was quantified. Values are expressed relative to the amount of biofilm obtained for *X. fastidiosa* in non-supplemented PD2 (considered 100%). The experiment was repeated three times, and each experiment contained three replicates. Data from a representative experiment are presented.

To test the efficiency and localized effects of the chelators, we determined the available Ca using the Fluo-5N, a fluorescent BAPTA derivative. Comparing the Fluo-5N reactive Ca in the media, we detected equal Ca levels in the PD2 and PD2 BAPTA/AM media (Table 1). This was as expected as the AM ester derivitization prevents Ca chelation until it is taken into cells.

However, we detected 10-fold decrease in available Ca based on Fluo-5N fluorescence in PD2 + EGTA medium. In PD2, PD2 + EGTA and PD2 + BAPTA/AM the concentration of total Ca measured by ICP-OES was equal, while the addition of 2 mM Ca was additive to the base media (Table 2-1). For comparison, the available reactive Ca was converted to concentration by creating a standard curve using a titration of CaCl₂ solution with Fluo-5N.

Table 2-1 Comparison of means (\pm SEM) of available and total calcium in various PD2 media.

| Treatment | Available Ca (RF: Ex 485nm Em 520 nm) (n=4) | Available Ca ¹ (mM) | ICP-OES ² (mM) |
|----------------|---|-----------------------------------|------------------------------|
| PD2 | 127 \pm 4 | 0.083 | 0.073 \pm 0.002 |
| PD2 + Ca | 2460 \pm 15 | 1.59 | 2.176 \pm 0.080 |
| PD2 + EGTA | 11 \pm 2 | 0.0073 | 0.074 \pm 0.002 |
| PD2 + BAPTA/AM | 136 \pm 4 | 0.088 | 0.074 \pm 0.002 |

¹Calculated from Fluo-5 reactivity with CaCl₂ standard solution in 10 mM Tris pH 7.0

² Inductively-coupled, plasma-optical emission spectroscopy was used to measure total calcium concentration

Analysis of intracellular and extracellular Ca in *X. fastidiosa* biofilm was achieved by using Fluo-5N/AM derivative and Fluo-5N. Intracellular Ca in cells cultured in PD2 + Ca was elevated 6-fold relative to PD2 alone, while BAPTA/AM treatment decreased available intracellular Ca ~2-fold (Table 2-2). This assay assumes relatively equal loading in cells, and microscopic analysis confirmed that fluorescence was a result of intracellular signal from *X. fastidiosa* (data not shown). Using cell impermeable Fluo-5N, the washed cells were assayed for extracellular Ca levels. The available extracellular Ca in EGTA media was decreased to 0.5-fold of that in PD2 medium, while BAPTA/AM extracellular was 1.2-fold higher than that in PD2 medium, and that with Ca-treated medium was 4-fold higher than that in PD2 medium (Table 2).

Table 2-2. Comparison of means (\pm SEM) of intracellular and extracellular Ca and total calcium in various PD2 media.

| Treatment | Extracellular Ca (RF: Ex 485nm Em 520 nm) (n=4) | Intracellular Ca (RF: Ex 485nm Em 520 nm) (n=4) |
|-----------------------------|---|---|
| PD2 | 86 \pm 8 | 12 \pm 1 |
| PD2 + Ca ¹ | 301 \pm 6 | 83 \pm 14 |
| PD2 + EGTA ² | 46 \pm 10 | 9 \pm 2 |
| PD2 + BAPTA/AM ³ | 104 \pm 7 | 5 \pm 1 |

¹ 2mM CaCl₂ was added to PD2

² 1.5mM of extracellular calcium-chelator EGTA (ethylene glycol tetraacetic acid) was added to PD2

³ 75 μ M of intracellular calcium-chelator BAPTA/AM (1, 2-bis (o-aminophenoxy) ethane-N, N, N', N'-tetraacetic acid acetoxymethyl ester) was added to PD2

Effect of Ca on autoaggregation and cell-to-cell attachment.

Autoaggregation has been previously defined in *X. fastidiosa* as the formation of spherical compact aggregates of cells attached to each other that precedes biofilm formation (De La Fuente et al., 2008). The autoaggregation process observed in microfluidic chambers was initiated at two days after inoculation of PD2 or 2 mM Ca supplemented media. Cells inoculated to PD2 media supplemented with 1.5 mM EGTA showed considerably less autoaggregation than the control media. Five days post-inoculation, the 2 mM Ca treatment had the highest level of autoaggregation followed by PD2, while almost no aggregation was observed in 1.5 mM EGTA treatment during this period (Fig 2-3A). We also observed that when Ca was added to the media, *X. fastidiosa* increased polar attachment to the substrate as opposed to lying flat against the glass surface. On average, approximately 60 % of cells grown in supplemented PD2 + Ca showed polar attachment, compared to control PD2 media where only 30% of the cells were in this position.

Analysis of cell settling rates, as a measurement of cell-to-cell aggregation, demonstrated that cells from media containing Ca showed significantly faster sedimentation rates ($0.31 \Delta OD_{600} \text{ min}^{-1}$) ($P \leq 0.0001$) than cells grown in non-supplemented media (mean = $0.15 \Delta OD_{600} \text{ min}^{-1}$), EGTA-containing media (mean = $0.08 \Delta OD_{600} \text{ min}^{-1}$), or BAPTA/AM-containing media (mean = 0.04). Similarly, significant differences were found between non-supplemented media and either EGTA-containing media ($P = 0.03$) or BAPTA/AM-containing media ($P = 0.003$). *X. fastidiosa* grown in media containing EGTA display a non-significant difference in settling rate compared to BAPTA/AM ($P = 0.212$) (Fig 2-3B).

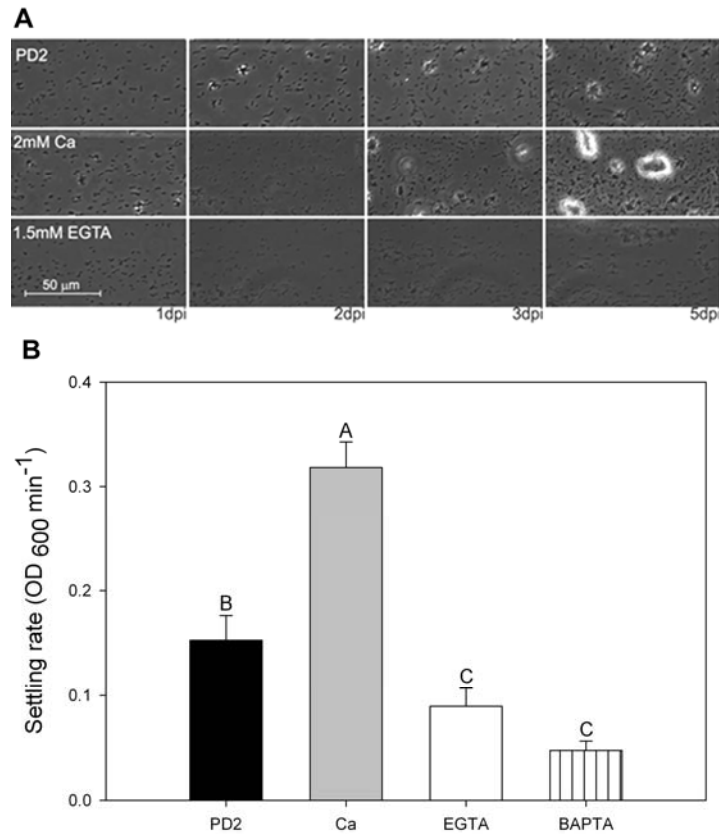


Figure 2-3. Evaluation of cell-to-cell aggregation of *X. fastidiosa* at different Ca concentrations.

A) Time-lapse micrographs showing formation of spherical compact autoaggregates inside microfluidic chambers. Images were captured at 1, 2, 3, and 5 dpi. B) Settling rates of *X.*

fastidiosa WT as a measure of cell-to-cell aggregation. Biofilms cells were obtained from growth cultures in PD2, PD2 + 2 mM Ca, PD2 + 1.5 mM EGTA, and 75 μ m BAPTA/AM. Different letters on the bars indicate significant differences according to the GLIMMIX procedure ($P \leq 0.05$). Error bars represent standard errors of the mean (n = 4).

Effect of calcium on cell adhesion force.

Adhesion force (AF) comparisons among WT *X. fastidiosa* cells in media containing 2 mM Ca (AF = 223 pN), 1.5 mM EGTA (AF = 190 pN), 75 μ M BAPTA/AM (AF = 179 pN), and non-supplemented PD2 media (AF = 160 pN) showed that the presence of Ca significantly increases the adhesion force of the cells compared with cells in non-supplemented PD2 ($P = 0.008$) and with cells treated with BAPTA/AM ($P = 0.05$) (Fig 2-4). In contrast, no significant differences were observed between the adhesion force of Ca and EGTA-treated cells ($P = 0.14$) or between the adhesion force of cells in non-supplemented PD2 and cells in EGTA-containing media ($P = 0.15$) and cells treated with BAPTA/AM ($P = 0.39$).

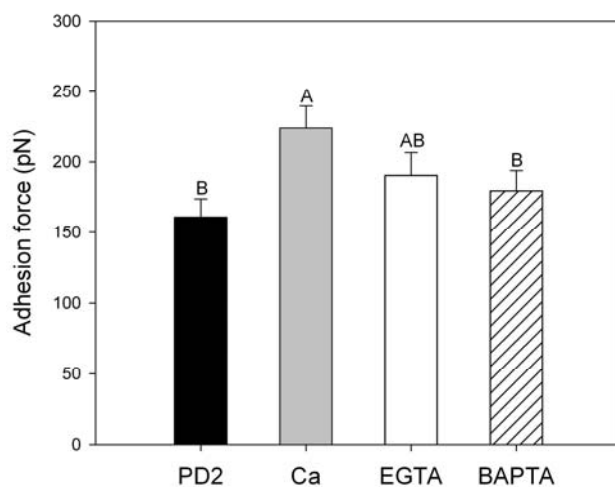


Figure 2-4 Adhesion force of *X. fastidiosa* WT strain in non-supplemented PD2, PD2 + 2 mM Ca, PD2 + 1.5 mM EGTA, and PD2 + 100 μ M BAPTA/AM assessed in microfluidic chambers. Different letters on the bars indicate significant differences according to the GLIMMIX procedure ($P \leq 0.05$). Error bars represent standard errors of the mean ($n = 7$).

Effect of calcium on tetracycline-treated cells.

Tetracycline was added to the media as a bacteriostatic agent to test the hypothesis that *de novo* protein synthesis is needed to observe the effect of Ca in attachment to surfaces. Evaluation of the adhesion force of tetracycline-treated cells indicated that, in Ca-supplemented media, the adhesion force of tetracycline-treated cells (AF = 172 pN) was significantly lower than the adhesion force of non-treated cells (AF = 223 pN) ($P = 0.029$) (Fig 2-5). Comparisons of the adhesion force of tetracycline-treated cells in Ca-supplemented media (AF= 172 pN) and non-supplemented media (AF =198 pN) indicated no significant differences ($P = 0.30$). These results suggest that protein synthesis is needed for Ca to have an effect on surface attachment of *X. fastidiosa*.

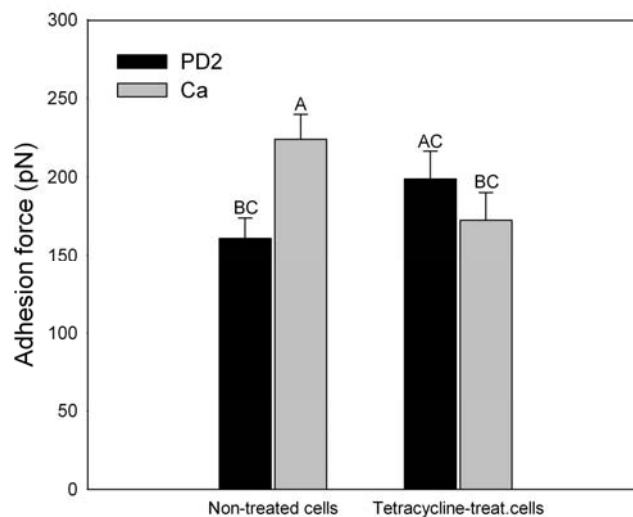


Figure 2-5. Adhesion force of tetracycline-treated and non-treated *X. fastidiosa* WT cells in Ca-supplemented and non-supplemented PD2.

Different letters on the bars indicate significant differences according to the GLIMMIX procedure ($P \leq 0.05$). Error bars represent standard errors of the mean ($n = 7$).

Effect of Ca on biofilm formation and adhesion force by *X. fastidiosa* attachment-defective mutants.

Biofilm formation assessments in 96-well plates of *X. fastidiosa* mutants indicated a positive correlation between the increase in the concentration of Ca and the formation of biofilm for *pilB*⁻ (type IV pili-defective) and *fimA*⁻ (type I pili defective) (Fig2-6A). In *fimA*⁻, biofilm production was increased by 600% at 2 mM Ca and 350% for *pilB*⁻ at 2.5 mM Ca. For the double mutant defective in type I and type IV pili (*fimA*⁻/*pilO*⁻), the addition of Ca did not significantly affect the formation of biofilm. Planktonic growth of the *X. fastidiosa* mutants was not affected by the range of Ca supplements tested (Figure 2-6B).

The microfluidic adhesion force assay indicated that the *pilB*⁻ mutant has significantly increased adhesion in the presence of 2 mM Ca (AF = 211 pN) compared with cells in control (non-supplemented) media (AF = 178 pN) ($P = 0.044$). No significant differences in adhesion force were found for *fimA*⁻ (AF = 130 pN) and *fimA*⁻/*pilO*⁻ (AF = 118pN) mutants in Ca supplemented media compared to control media (AF = 125 pN and 113 pN, respectively) ($P = 0.653$) (Fig 2-6C).

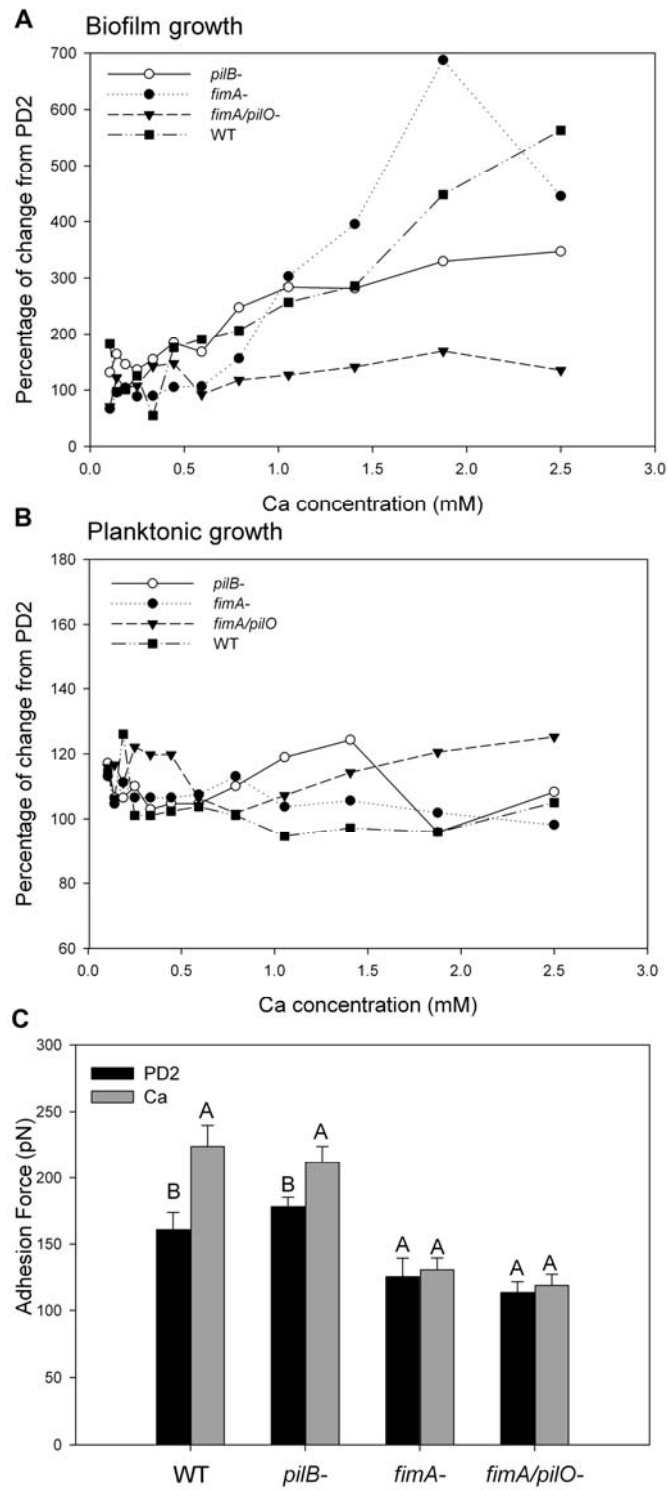


Figure 2-6. Effects of Ca on biofilm formation, growth, and adhesion force of *X. fastidiosa* mutant strains defective in surface structures.

A) Biofilm formation in the presence of Ca (0.1 mM to 2.5 mM). Values are expressed relative to the amount of biofilm obtained for *X. fastidiosa* in non-supplemented PD2 (considered 100%). The experiment was repeated three times; and data from a representative experiment is presented. B) Corresponding planktonic growth under the same conditions as (A). C) Adhesion force in PD2 media and in PD2 supplemented with 2 mM Ca as measured inside microfluidic chambers. Different letters on the bars indicate significant differences according to the GLIMMIX procedure ($P \leq 0.05$). Error bars represent standard errors of the mean ($n = 6$).

Exopolysaccharides production in the presence of calcium.

An indirect measurement of EPS production was performed using a phenol-sulfuric acid assay. This assay quantifies total sugars in glucose equivalents and used as an indicator of EPS production. The sugar content per cell in biofilm from PD2 and PD2 supplemented with 2 mM Ca or 1.5 mM EGTA was not significantly different ($P = 0.696$) (Fig. 2-7A), regardless of the amount of biofilm observed among the treatments (Fig 2-7B).

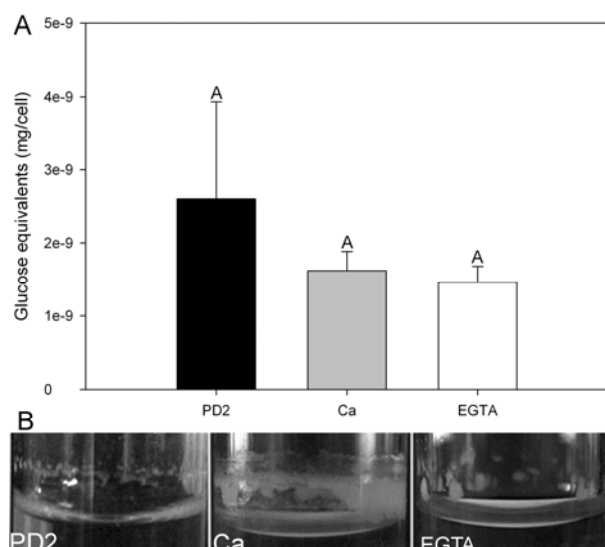


Figure 2-7. EPS produced by biofilm cells grown in non-supplemented PD2 and PD2 supplemented with 2 mM Ca and 1.5 mM EGTA.

A) EPS measurement in cell forming biofilm quantified in the amount of glucose equivalents. Different letters on the bars indicate statistically significant differences according to the GLIMMIX procedure ($P \leq 0.05$). Error bars represent standard errors of the mean ($n = 10$). B) Images of biofilm formed on glass surface of Erlenmeyer flasks with cultures of *X. fastidiosa* WT strain under different concentrations of Ca.

Effect of Ca on biofilm three-dimensional structure.

Examination of Z-stacks' three-dimensional images and measurements of biofilm thickness indicated that *X. fastidiosa* biofilm cells grown in 2 mM Ca-supplemented media exhibited a significantly thicker biofilm (mean = 31.04 μm) compared with media containing 1.5 mM EGTA (mean = 9.95 μm) and control non-supplemented media (mean = 12.93 μm) ($P \leq 0.001$).

Similarly, significant differences were observed between EGTA and control non-supplemented

media ($P \leq 0.001$) (Fig. 2-8A). Two and three-dimensional observations of the biofilm obtained among the treatments also showed differences in cells' distribution and orientation (Fig. 2-8 B-F). Biofilm obtained from cells in the presence of Ca was continuous and compact throughout the slide, and cells exhibited a vertical polar orientation (Fig. 2-8 D, E). Similarly, in non-supplemented media, the biofilm was formed as a continuous structure; however, cell-to-cell spaces were bigger, and cells were horizontally oriented (Fig. 2-8 B, C). In EGTA-containing media, biofilm was formed as discontinuous clusters of cells exhibiting the largest cell-to-cell separations (Fig. 8 F, G).

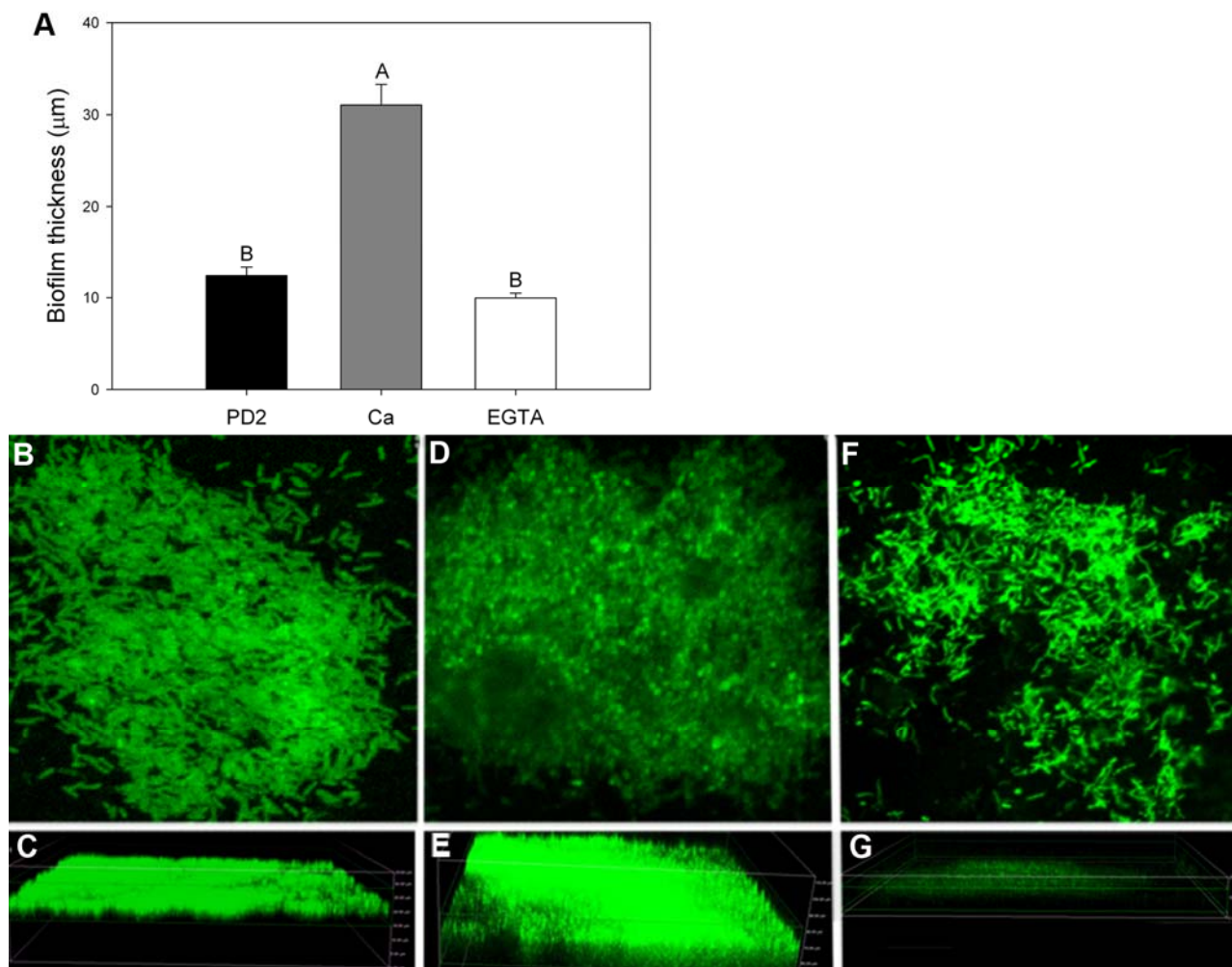


Figure 2-8. Biofilm architecture, confocal scanning microscopy images of biofilm formed on microscope slides.

A) Biofilm thickness measurements of biofilm formed in the condition mentioned below. B-C) Two and three-dimensional images of biofilm formed in PD2 non supplemented media. D-E) Two and three-dimensional images of biofilm formed in PD2 supplemented with 2 mM Ca. F-G) Two and three-dimensional images of biofilm formed in PD2 containing 1.5 mM EGTA. Different letters on the bars indicate significant differences according to the GLIMMIX procedure ($P \leq 0.05$). Error bars represent standard errors of the mean ($n = 9$).

Effect of calcium on cell twitching motility. The effect of Ca on cell motility, as determined by the colony fringe width on agar plates, indicated that colonies grown on PW agar supplemented with 2 mM Ca (mean = 123.55 μm) have a 1.5-fold increase in fringe size compared to colonies grown on plain PW (mean = 80.65 μm) ($P \leq 0.001$) and an 11-fold increase compared with colonies grown on PW supplemented with 1.5 mM EGTA (mean = 11.40 μm) ($P \leq 0.001$) (Fig 2-9A-B).

The movement of the cells quantified in the microfluidic chambers correlates with the results obtained from the colony fringe observations. In the presence of Ca, cells moved at higher speeds (mean = 0.53 $\mu\text{m min}^{-1}$) compared to those in PD2 (mean = 0.32 $\mu\text{m min}^{-1}$) ($P \leq 0.001$) and PD2 supplemented with 1.5 mM EGTA (mean = 0.044 $\mu\text{m min}^{-1}$) ($P \leq 0.001$) (Fig 9C).

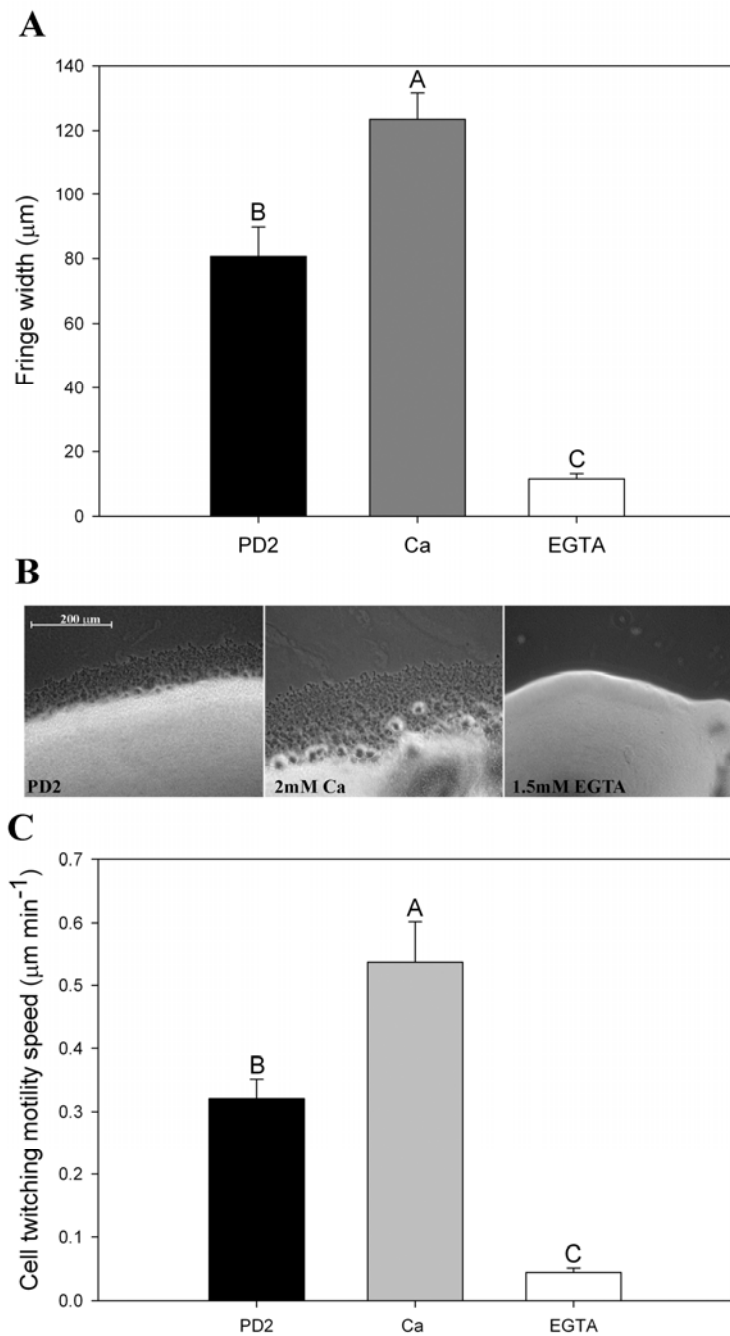


Figure 2-9. Effects of Ca on *X. fastidiosa* cell twitching motility.

A) Colony fringe width of *X. fastidiosa* WT strain cultured on PW agar and PW supplemented with 2 mM Ca and 1.5 mM EGTA. Different letters on the bars indicate statistically significant differences according to the GLIMMIX procedure ($P \leq 0.05$). Error bars represent standard

errors of the mean ($n = 10$). B) Representative micrographs of colony fringe on agar plates in the treatments mentioned in (A). C) Twitching speed assessments of cells incubated in PD2 and PD2 supplemented with 2 mM Ca or 1.5 mM EGTA in microfluidic chambers. Different letters on the bars indicate significant differences according to the GLIMMIX procedure ($P \leq 0.05$). Error bars represent standard errors of the mean ($n = 10$).

Discussion

Availability of mineral nutrients can act as a stimuli, eliciting changes in bacteria that can promote the establishment and development of biofilms in plant- (Danhorn and Fuqua, 2007) and animal- (Prakash et al., 2003) associated bacteria. Metals are important constituents of the xylem sap, one of the natural environments of *X. fastidiosa* (Peuke, 2000). Therefore, we hypothesized that variation in metals would have an impact on *X. fastidiosa* colonization and biofilm formation. Calcium levels specifically affected biofilm production, so we focused on refining the role of Ca on *X. fastidiosa* biofilm formation.

Divalent cations, including Ca and Mg, have been previously implicated in the accumulation of biofilm by different bacteria. Primarily, these cations were suspected to act as cross-bridging molecules that increase biofilm matrix stability (Rose, 2000; Chen and Stewart, 2002). However, they also play a role in regulation of bacterial gene expression related to virulence factors and biofilm formation (Patrauchan et al., 2005; Sarkisova et al., 2005). For example, physiological changes related to adhesion have been demonstrated in marine *Pseudoalteromonas* sp., where Ca influences the production of extracellular matrix material associated with biofilm (Sarkisova et al., 2005), and in *Pseudomonas putida*, where the presence of Ca-binding membrane proteins are

involved in cell attachment to seeds (Espinosa-Urgel et al., 2000). In *Erwinia carotovora* high levels of Ca repress the expression of PehA, an endopolygalacturonase that is one of its main virulence determinants (Flego et al., 1997). The opposite effect, i.e. enhancement of virulence, has been seen in *Pseudomonas aeruginosa* where addition of Ca increases the production of extracellular proteases as well as increases the biofilm thickness by enhancing the expression of the alginate biosynthetic genes, a main constituent of the extracellular matrix (Sarkisova et al., 2005). Results from this study show that Ca is involved in the regulation of *X. fastidiosa* biofilm formation, cell surface attachment, and twitching motility.

Bacterial attachment to surfaces is important in the early steps of biofilm development. Biofilm formation begins with transient attachment of cells, followed by stable attachment to surfaces. In advanced stages, cells increase the production of EPS to create a matrix that holds together the mature biofilm (Sauer et al., 2002; Hinsa et al., 2003; Sarkisova et al., 2005). Experiments from the present study indicate that addition of Ca significantly increases the attachment strength of the cells to the surface. Investigations into the mechanism of *X. fastidiosa* cell adhesion using X-ray microanalyses of occluded citrus xylem vessels led Leite et al. (2002) to propose a model where Ca and Mg ions act as cross-binding molecules that create a bridge between the negatively-charged xylem vessels and the negatively-charged bacterial EPS. According to this model, extracellular calcium is responsible for cell attachment acting as an external physical force. Our study shows that, in addition to the “bridging” effect that can occur with Ca, a metabolic-dependent effect is also responsible for Ca influence on biofilm and movement.

The transient exposure of *X. fastidiosa* to extracellular chelator EGTA did not produce a considerable reduction in adhesion force compared to Ca-treated cells. Only after continuous culturing in EGTA (e.g. in the crystal violet assay) did we see strong effects. This discrepancy could be explained by differences in the incubation time of these systems and the static versus dynamic nature of the experiments. In the microtiter plate assay, cells incubated for 5 days have to overcome several division cycles under low Ca conditions in the same batch of media contained in the well, thus new cells should contain lower amount of intracellular Ca. By contrast, in the microfluidic chamber assays, cells are exposed to reduced Ca concentration just for the length of the experiment (few hours), and the media is being constantly replenished inside the channels. However, the fact that cells treated with BAPTA/AM showed a significant reduction in the adhesion force when compared with Ca treated cells, reduced cell-to-cell adhesion, and produced significantly less biofilm in the static assay strongly suggests that other mechanisms that require intracellular Ca are contributing to the formation of biofilm.

The measurements of adhesion force in tetracycline-treated *X. fastidiosa* further corroborate the regulatory effect of Ca on cell adhesion. Since *de novo* protein synthesis is compromised by tetracycline treatment, which inhibits bacterial protein synthesis by preventing tRNA union with the ribosome (Chopra and Roberts, 2001), any response to Ca under these conditions would be more strongly associated with physical cell adhesion. No differences were found in the adhesion force between translation arrested cells in Ca-supplemented versus non-supplemented PD2 media, indicating that the effect of Ca in increasing adhesion force requires cells to be metabolically active. Moreover, the results obtained with the double mutant *fimA/pilO*⁻ missing type I and type IV pili support this hypothesis. The double mutant did not respond to Ca in static

cultures, when biofilm was quantified in microtiter plates, and Ca did not change the adhesion force to a substrate in microfluidic chambers. If the effect of Ca was purely due to extracellular electrostatic interaction at the cell-substrate surface level, this effect should have been observed with this mutant. The role of Ca in biofilm formation and specifically in cell attachment requires metabolically active cells and the presence of type I and type IV pili.

Static biofilm production by *X. fastidiosa* pili-mutants *pilB*⁻ and *fimA*⁻ (type I- and type IV-defective mutants, respectively) responded to Ca addition. But only the type IV pili mutant (*pilB*⁻) responded by increasing the adhesion force to a substrate. These findings suggest that the strongest increase in attachment is due to an influence of Ca on type I pili. Previously, type I pili were shown to be responsible for the strongest attachment to surfaces (11). The role of Ca in type IV pili could be related to the primary function of these fimbriae that assist in cell twitching motility. The speed of the cell movements and colony fringe width demonstrate that the presence of high concentrations of Ca significantly increases the twitching movement of the cells.

Although cell movement and cell adhesion seem functionally opposite, in early stages of biofilm formation, twitching helps the bacteria colonize or in later stages decolonize one area and re-colonize another. Additionally, even though type IV pili are mainly responsible for cell motility, they are able to contribute to cell adhesion.

The function of Ca in bacterial motility via type IV pili has been documented in *Pseudomonas aeruginosa*. Binding and release of Ca by a PilY1calcium binding site similar to that seen in the canonical EF-hand Ca-binding, located in the C-terminal domain of the protein, elicit extension and retraction of the type IV pili required for twitching motility (Orans et al., 2010). A

homologue of the *P. aeruginosa* *pilY1* gene is present in *X. fastidiosa*. PilY1 is a minor protein predicted to be associated with the tip of type IV pili (Mattick, 2002; Li et al., 2006; Li et al., 2007). In *X. fastidiosa*, the exact molecular mechanism(s) that regulate twitching motility have not been investigated, and so far no previous reports have found evidence of a role of Ca on type I pili regulation in *X. fastidiosa* or in other bacteria.

EPS production is one of the main factors thought to influence the rate and degree of attachment of microbial cells to different surfaces and to each other (Dharmapuri and Sonti, 1999). Ca has been implicated in the regulation of bacterial gene expression eliciting the production of EPS in *Pseudoalteromonas* sp. (Patrauchan et al., 2005). Analysis of the *X. fastidiosa* EPS production shows that media containing Ca (PD2 and PD2 supplemented with additional Ca) produce equal amounts of EPS compared to cells grown in media where Ca is chelated by EGTA. This result suggests that Ca does not affect the production of EPS by *X. fastidiosa* cells. In *X. fastidiosa* the quorum-sensing mechanism mediated by a diffusible signal factor (DSF) is involved in the regulation of EPS production (Scarpari et al., 2003). We anticipated that production of EPS should be dependent on the population density attached to the substrate, which is higher in the presence of Ca. However, with the technique used in our studies we were unable to find differential EPS production that correlated with changing Ca levels.

Another step in the formation of biofilm is the aggregation of cells after initial attachment. Our experiments showed that, in the presence of exogenous Ca, cells exhibit taller biofilm aggregates, formation occurs at a faster rate, and attachment to surfaces and to other cells is stronger. Adhesion and aggregation of cells are responsible for the final architecture of biofilm.

Our confocal observations of biofilm formed on microscope slides showed a polar orientation of the cells in Ca supplemented treatments. Similar observations were made by Killiny and Almeida (2009) on the biofilm formed in the foregut of the insect vector. In their experiments, the biofilm formation process includes initial lateral attachment followed by polar attachment possibly by the action of the type I pili to increase the surface area of nutrient absorption. The mediation of the polar attachment may also be enhanced by the effect of Ca on this fimbrial structure.

It should be noted that previous studies have shown that the chemical composition of the growth media and xylem sap influence *X. fastidiosa* aggregation and biofilm formation (Andersen et al., 2007; Zaini et al., 2009). Cheng et al. (2009) found that PW media supplemented with xylem sap of PD-susceptible grapevine induced increased bacterial aggregation and biofilm formation compared to media supplemented with sap of PD-resistant varieties. Andersen et al. (2007) analyzed the relationship between sap chemical components and biofilm formation and compared the chemical composition of PD-susceptible and PD-resistant cultivars. Increasing concentrations of Ca, Mg, and amino acids, from the PD-resistant level to that present in the PD-susceptible sap, correlated with an increase in cell aggregation and improved planktonic growth. However, this is the first study to focus solely on Ca as the stimulatory factor.

The present study shows that Ca has multiple roles during *in vitro* biofilm formation by *X. fastidiosa*. Ca appears to influence biofilm formation by both extracellular ionic bridging and by intracellular stimulation that requires protein synthesis. Physiological changes in response to Ca are increased cell attachment most likely via type I pili, increased twitching motility, and increased cell-to-cell attachment responsible for aggregation. Ca affects the initial stages of

biofilm development mainly related to the cell attachment and has a less prominent role in biofilm maturation at later stages. *In vivo* experiments that correlate the nutritional status of the plant with the development of the disease and expression analyses of *X. fastidiosa* in the presence of Ca are necessary to further elucidate the molecular mechanisms involved in Ca promotion of biofilm formation.

Acknowledgements

This project was supported by the Agriculture and Food Research Initiative Competitive Grants Program Grant no. 2010-65108-20633 from the USDA National Institute of Food and Agriculture and the Alabama Agricultural Experiment Station (AAES) Hatch Grant Program. We thank Harvey C. Hoch (Cornell University) and Tom Burr (Cornell University) for providing the *X. fastidiosa* mutants strains and Steven Lindow (University of California, Berkley) for providing the *X. fastidiosa* GFP-expressing strain. We acknowledge the Auburn University Confocal Facilities for the use of their microscope. We also acknowledge the assistance of Melody Smith in initial experiments with Fluo-5.

References

- Andersen, P.C., Brodbeck, B.V., Oden, S., Shriner, A., and Leite, B. 2007. Influence of xylem fluid chemistry on planktonic growth, biofilm formation and aggregation of *Xylella fastidiosa*. *Fems Microbiology Letters* 274:210-217.
- Chang, C.J., Donaldson, R., Brannen, P., Krewer, G., and Boland, R. 2009. Bacterial leaf scorch, a new blueberry disease caused by *Xylella fastidiosa*. *Hortscience* 44:413-417.
- Chatterjee, S., Newman, K.L., and Lindow, S.E. 2008. Cell-to-cell signaling in *Xylella fastidiosa* suppresses movement and xylem vessel colonization in grape. *Mol. Plant Microbe Interact.* 21:1309-1315.
- Chen, X., and Stewart, P.S. 2002. Role of electrostatic interactions in cohesion of bacterial biofilms. *Applied Microbiology and Biotechnology* 59:718-720.
- Cheng, D.W., Lin, H., Walker, M.A., Stenger, D.C., and Civerolo, E.L. 2009. Effects of grape xylem sap and cell wall constituents on in vitro growth, biofilm formation and cellular aggregation of *Xylella fastidiosa*. *European Journal of Plant Pathology* 125:213-222.
- Chopra, I., and Roberts, M. 2001. Tetracycline antibiotics: mode of action, applications, molecular biology, and epidemiology of bacterial resistance. *Microbiology and Molecular Biology Reviews* 65:232-+.
- Costerton, J.W., Lewandowski, Z., Caldwell, D.E., Korber, D.R., and Lappinscott, H.M. 1995. Microbial biofilms. *Annu Rev Microbiol* 49:711-745.
- Danhorn, T., and Fuqua, C. 2007. Biofilm formation by plant-associated bacteria. *Annu Rev Microbiol* 61:401-422.
- Dasgupta, N., Ashare, A., Hunninghake, G.W., and Yahr, T.L. 2006. Transcriptional induction of the *Pseudomonas aeruginosa* type III secretion system by low Ca^{2+} and host cell contact proceeds through two distinct signaling pathways. *Infection and Immunity* 74:3334-3341.
- Davis, M.J., Purcell, A.H., and Thomson, S.V. 1980. Isolation media for the Pierce's disease bacterium. *Phytopathology* 70:425-429.
- Davis, M.J., Whitcomb, R.F., and Gillaspie, A.G.J. 1981. Fastidious bacteria of plant vascular tissue and invertebrates (including so called rickettsia-like bacteria). Pages 2172-2188 in: *The prokaryotes : a handbook on habitats, isolation, and identification of bacteria*, M.P. Starr, H. Stolp, H.G. Truper, A. Balows, and H.G. Schlegel, eds. Springer-Verlag, Berlin ; New York.
- De La Fuente, L., Burr, T.J., and Hoch, H.C. 2007a. Mutations in type I and type IV pilus biosynthetic genes affect twitching motility rates in *Xylella fastidiosa*. *J Bacteriol* 189:7507-7510.
- De La Fuente, L., Burr, T.J., and Hoch, H.C. 2008. Autoaggregation of *Xylella fastidiosa* cells is influenced by type I and type IV pili. *Appl. Environ. Microbiol.* 74:5579-5582.
- De la Fuente, L., Montanes, E., Meng, Y.Z., Li, Y.X., Burr, T.J., Hoch, H.C., and Wu, M.M. 2007b. Assessing adhesion forces of type I and type IV pili of *Xylella fastidiosa* bacteria by use of a microfluidic flow chamber. *Appl Environ Microbiol* 73:2690-2696.
- DeBord, K.L., Galanopoulos, N.S., and Schneewind, O. 2003. The *ttsA* gene is required for low-calcium-induced type III secretion of Yop proteins and virulence of *Yersinia enterocolitica* W22703. *Journal of Bacteriology* 185:3499-3507.
- Dharmapuri, S., and Sonti, R.V. 1999. A transposon insertion in the gumG homologue of *Xanthomonas oryzae* pv. *oryzae* causes loss of extracellular polysaccharide production and virulence. *Fems Microbiology Letters* 179:53-59.

- Doyle, J., and Doyle, J.L. 1987. Genomic plant DNA preparation from fresh tissue - CTAB method. *Phytochem. Bull.* 19:11-15.
- Dubois, M.G., Gilles, K.A., Hamilton, J.K., Rebers, P.A., Smith, F., and 1956. Colorimetric method of determination of sugars and related substances *Analytical Chemistry* 28:350-354.
- Espinosa-Urgel, M., Salido, A., and Ramos, J.L. 2000. Genetic analysis of functions involved in adhesion of *Pseudomonas putida* to seeds. *Journal of Bacteriology* 182:2363-2369.
- Flego, D., Pirhonen, M., Saarilahti, H., Palva, T.K., and Palva, E.T. 1997. Control of virulence gene expression by plant calcium in the phytopathogen *Erwinia carotovora*. *Molecular Microbiology* 25:831-838.
- Francis, M., Lin, H., Cabrera-La Rosa, J., Doddapaneni, H., and Civerolo, E.L. 2006. Genome-based PCR primers for specific and sensitive detection and quantification of *Xylella fastidiosa*. *European Journal of Plant Pathology* 115:203-213.
- Guilhabert, M.R., and Kirkpatrick, B.C. 2005. Identification of *Xylella fastidiosa* antivirulence genes: hemagglutinin adhesins contribute to *Xylella fastidiosa* biofilm maturation and colonization and attenuate virulence. *Molecular Plant-Microbe Interactions* 18:856-868.
- Hall-Stoodley, L., Costerton, J.W., and Stoodley, P. 2004. Bacterial biofilms: from the natural environment to infectious diseases. *Nature Reviews Microbiology* 2:95-108.
- Hansch, R., and Mendel, R.R. 2009. Physiological functions of mineral micronutrients (Cu, Zn, Mn, Fe, Ni, Mo, B, Cl). *Curr. Opin. Plant Biol.* 12:259-266.
- Hinsa, S.M., Espinosa-Urgel, M., Ramos, J.L., and O'Toole, G.A. 2003. Transition from reversible to irreversible attachment during biofilm formation by *Pseudomonas fluorescens* WCS365 requires an ABC transporter and a large secreted protein. *Molecular Microbiology* 49:905-918.
- Hopkins, D.L., and Purcell, A.H. 2002. *Xylella fastidiosa*: cause of Pierce's disease of grapevine and other emergent diseases. *Plant Disease* 86:1056-1066.
- Kierck, K., and Watnick, P.I. 2003. The *Vibrio cholerae* O139O-antigen polysaccharide is essential for Ca²⁺-dependent biofilm development in sea water. *Proceedings of the National Academy of Sciences of the United States of America* 100:14357-14362.
- Killiny, N., and Almeida, R.P.P. 2009. *Xylella fastidiosa* afimbrial adhesins mediate cell transmission to plants by leafhopper vectors. *Appl Environ Microbiol* 75:521-528.
- Lecourieux, D., Ranjeva, R., and Pugin, A. 2006. Calcium in plant defence-signaling pathways. *New Phytologist* 171:249-269.
- Leite, B., Ishida, M.L., Alves, E., Carrer, H., Pascholati, S.F., and Kitajima, E.W. 2002. Genomics and X-ray microanalysis indicate that Ca²⁺ and thiols mediate the aggregation and adhesion of *Xylella fastidiosa*. *Brazilian Journal of Medical and Biological Research* 35:645-650.
- Li, Y., Hao, G., Galvani, C.D., Meng, Y., De La Fuente, L., Hoch, H.C., and Burr, T.J. 2007. Type I and type IV pili of *Xylella fastidiosa* affect twitching motility, biofilm formation and cell-cell aggregation. *Microbiology* 153:719-726.
- Li, Y.X., Hao, G.X., Meng, Y.Z., Galvani, C.D., Hoch, H.C., and Burr, T.J. 2006. Genes required for twitching motility in *Xylella fastidiosa*. *Phytopathology* 96:S180-S180.
- Maathuis, F.J.M. 2009. Physiological functions of mineral macronutrients. *Curr. Opin. Plant Biol.* 12:250-258.
- Mattick, J.S. 2002. Type IV pili and twitching motility. *Annu Rev Microbiol* 56:289-314.

- Meng, Y.Z., Li, Y.X., Galvani, C.D., Hao, G.X., Turner, J.N., Burr, T.J., and Hoch, H.C. 2005. Upstream migration of *Xylella fastidiosa* via pilus-driven twitching motility. *Journal of Bacteriology* 187:5560-5567.
- Newman, K.L., R. P. P. Almeida, A. H. Purcell, and S. E. Lindow. 2003. Use of a green fluorescent strain for analysis of *Xylella fastidiosa* colonization of *Vitis vinifera*. *Appl Environ Microbiol* 69:7319-7327.
- Nhieu, G.T., Clair, C., Grompone, G., and Sansonetti, P. 2004. Calcium signalling during cell interactions with bacterial pathogens. *Biol. Cell* 96:93-101.
- O'Toole, G.A., Pratt, L.A., Watnick, P.I., Newman, D.K., Weaver, V.B., and Kolter, R. 1999. Genetic approaches to study of biofilms. *Biofilms* 310:91-109.
- Orans, J., Johnson, M.D.L., Coggan, K.A., Sperlazza, J.R., Heiniger, R.W., Wolfgang, M.C., and Redinbo, M.R. 2010. Crystal structure analysis reveals *Pseudomonas* PilY1 as an essential calcium-dependent regulator of bacterial surface motility. *Proceedings of the National Academy of Sciences of the United States of America* 107:1065-1070.
- Patrauchan, M.A., Sarkisova, S., Sauer, K., and Franklin, M.J. 2005. Calcium influences cellular and extracellular product formation during biofilm-associated growth of a marine *Pseudoalteromonas* sp. *Microbiology-Sgm* 151:2885-2897.
- Peuke, A.D. 2000. The chemical composition of xylem sap in *Vitis vinifera* L. cv. riesling during vegetative growth on three different franconian vineyard soils and as influenced by nitrogen fertilizer. *American Journal of Enology and Viticulture* 51:329-339.
- Prakash, B., Veeregowda, B.M., and Krishnappa, G. 2003. Biofilms: a survival strategy of bacteria. *Current Science* 85:1299-1307.
- Purcino, R.P., Medina, C.L., Martins, D., Winck, F.V., Machado, E.C., Novello, J.C., Machado, M.A., and Mazzafera, P. 2007. *Xylella fastidiosa* disturbs nitrogen metabolism and causes a stress response in sweet orange *Citrus sinensis* cv. Pera. *J. Exp. Bot.* 58:2733-2744.
- Ramey, B.E., Koutsoudis, M., von Bodman, S.B., and Fuqua, C. 2004. Biofilm formation in plant-microbe associations. *Current Opinion in Microbiology* 7:602-609.
- Rodrigues, C.M., Takita, M.A., Coletta-Filho, H.D., Olivato, J.C., Caserta, R., Machado, M.A., and de Souza, A.A. 2008. Copper resistance of biofilm cells of the plant pathogen *Xylella fastidiosa*. *Applied Microbiology and Biotechnology* 77:1145-1157.
- Romantschuk, M. 1992. Attachment of plant pathogenic bacteria to plant-surfaces. *Annual Review of Phytopathology* 30:225-243.
- Rose, R.K. 2000. The role of calcium in oral streptococcal aggregation and the implications for biofilm formation and retention. *Biochimica Et Biophysica Acta-General Subjects* 1475:76-82.
- Sarkisova, S., Patrauchan, M.A., Berglund, D., Nivens, D.E., and Franklin, M.J. 2005. Calcium-induced virulence factors associated with the extracellular matrix of mucoid *Pseudomonas aeruginosa* biofilms. *Journal of Bacteriology* 187:4327-4337.
- Sauer, K., Camper, A.K., Ehrlich, G.D., Costerton, J.W., and Davies, D.G. 2002. *Pseudomonas aeruginosa* displays multiple phenotypes during development as a biofilm. *Journal of Bacteriology* 184:1140-1154.
- Scarpari, L.M., Lambais, M.R., Silva, D.S., Carraro, D.M., and Carrer, H. 2003. Expression of putative pathogenicity-related genes in *Xylella fastidiosa* grown at low and high cell density conditions in vitro. *Fems Microbiology Letters* 222:83-92.

- Snyder, L., and Champness, W. 2003. Molecular genetics of bacteria. ASM Press, Washington, D.C.
- Stevenson, J.F., Matthews, M.A., and Ros, T.L. 2004. Grapevine susceptibility to Pierce's disease I: relevance to hydraulic architecture. American Journal of Enology and Viticulture 55:228-237.
- Yang, M., Cobine, P.A., Molik, S., Naranuntarat, A., Lill, R., Winge, D.R., and Culotta, V.C. 2006. The effects of mitochondrial iron homeostasis on cofactor specificity of superoxide dismutase 2. Embo J. 25:1775-1783.
- Zaini, P.A., De La Fuente, L., Hoch, H.C., and Burr, T.J. 2009. Grapevine xylem sap enhances biofilm development by *Xylella fastidiosa*. Fems Microbiology Letters 295:129-134.
- Zaini, P.A., Fogaca, A.C., Lupo, F.G.N., Nakaya, H.I., Vencio, R.Z.N., and da Silva, A.M. 2008. The iron stimulon of *Xylella fastidiosa* includes genes for type IV pilus and colicin V-like bacteriocins. Journal of Bacteriology 190:2368-2378.

Appendix

Analysis of *X. fastidiosa* exopolysaccharide production by biofilm-forming cells

Expression of genes requires for exopolysaccharide production

The expression of two genes involved in **exopolysaccharide** (EPS) synthesis (*gumD*, and *gumH*) were analyzed by RT-qPCR using primers *gumD*_F GGCGTTGCATGTAATGCTGGATCA, *gumD*_R GTGCTCAAATTTTCGCTCGAT, Probe TAACTGTTGTGCTGAGCCGCATGA and *gumH*_F ATCAATCCAGCACTCATCGCCTCA, *gumH*_R ATTGTAGTCCGTCACAGTCGCAGT and Probe AAACCCTCATGCCGCGACAGTGAAAT. Fifty milliliter conical tubes containing two glass slides and 10 ml of PD2 or PD2 supplemented with 2mM Ca Cl₂ or 1.5mM EGTA were inoculated with a 10µl of a suspension of cells and incubated at 28°C and 150 rpm for 7 days. Biofilms formed at the liquid-air interface of the slides were scraped off, and RNA was extracted using the RNA Shield™ Purification Kit (Zymo Research, Austin, TX) according to the manufacturer's instructions. Residual DNA was digested by incubation with 2 Units of OPTIZYME DNase I (Fisher Scientific, Pittsburg, PA) (45minutes at 37°C followed by 10 minutes at 65°C). RNA concentration was determined with a NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA) by measuring the absorbance at 260 nm, and 40 ng RNA were used to synthesize cDNA by reverse transcription using the Maxima First Strand cDNA Synthesis Kit for RT-qPCR (Thermo Scientific, Waltham, MA) according to manufacturer's instructions. 1.5 µl of cDNA were used for qPCR. Fold change in gene expression was calculated by the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen, 2001). The *dnaQ* gene was used as

the endogenous control to normalize gene expression. Other tested genes (*gyrB*, *nuoA*, *merD*) were not suitable as endogenous controls of gene expression under our experimental conditions (data not shown). Three replicates were used for each treatment. Two independent experiments were performed.

Exopolysaccharide visualization by laser scanning confocal microscopy (LSCM).

X. fastidiosa strain KLN59.3 modified with the insertion of green fluorescent protein (GFP) (Newman, 2003) was inoculated in 50 ml conical tubes containing 10 ml of PD2 media and PD2 supplemented with 2mM CaCl₂ or 1.5mM EGTA. Tubes were incubated at 28°C and 150 rpm for 13 days. Biofilm formed on the glass slides and were stained with calcofluor white (MP Biomedicals, Illkirch, France) to stain polysaccharides as previously described (Clark et al., 2007). Calcofluor white stains beta-linked polysaccharides (Wood, 1980; Wood et al., 1983; Clark et al., 2007) and Xf EPS have been shown to have both beta and alpha-linked polysaccharides (da Silva et al., 2001). The biofilms were observed with a Nikon Eclipse A1 confocal laser scanning inverted microscope (Nikon, Melville, NY) using a 60X oil immersion objective and the appropriate filters. Images were acquired with a CoolSNAP HQ2 camera (Photometrics, Tucson, AZ) and processed with NIS-Elements AR software, version 3.0 (Nikon, Melville, NY). Two independent experiments were performed.

Results

Expression of genes involved in EPS biosynthesis under replete and depleted Ca conditions.

Analysis of the gene expression of *X. fastidiosa* biofilms formed on glass slides indicated that the supplementation of Ca to the media results in the up-regulation of the two genes tested *gumD* (fold change: 2.44 ± 0.21) and *gumH* (fold change: 2.25 ± 0.43) compared with the non-supplemented PW media. In contrast, chelation of Ca with EGTA results in a significant decrease in the expression of both genes tested, *gumD* (0.13 ± 0.014) and *gumH* (fold change: 0.11 ± 0.01) (Fig. 2-10)

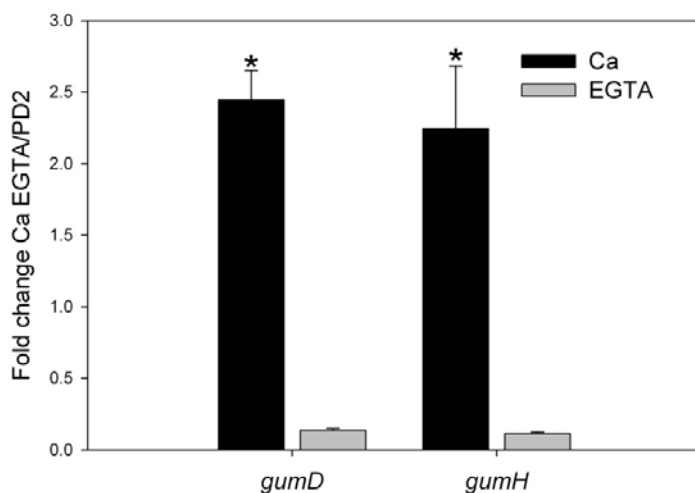


Figure 2-10. Gene expression analysis of *gumD* and *gumH* in *X. fastidiosa* biofilm formed on microscope slides.

Results are expressed as the fold change in gene expression in Ca and EGTA treatments, considering PD2 as the basal condition. Asterisks indicate significant differences in gene expression as determined by the t-test (fold change >1 , $P < 0.05$).

Exopolysaccharide distribution in biofilms

Biofilms grown in PD2 showed a heterogeneous structure, with cells being oriented horizontally, and EPS (stained bluish-white by calcofluor white) was observed in patches, intermixed with some cell groups, or as loose clumps that surrounded cell masses. In the EGTA treatment, EPS is very scarce and observed as a layer on the cells surface. In the Ca treatment, the abundant EPS is covering the continuous biofilm, forming a matrix in which cells are embedded (Fig 2-11).

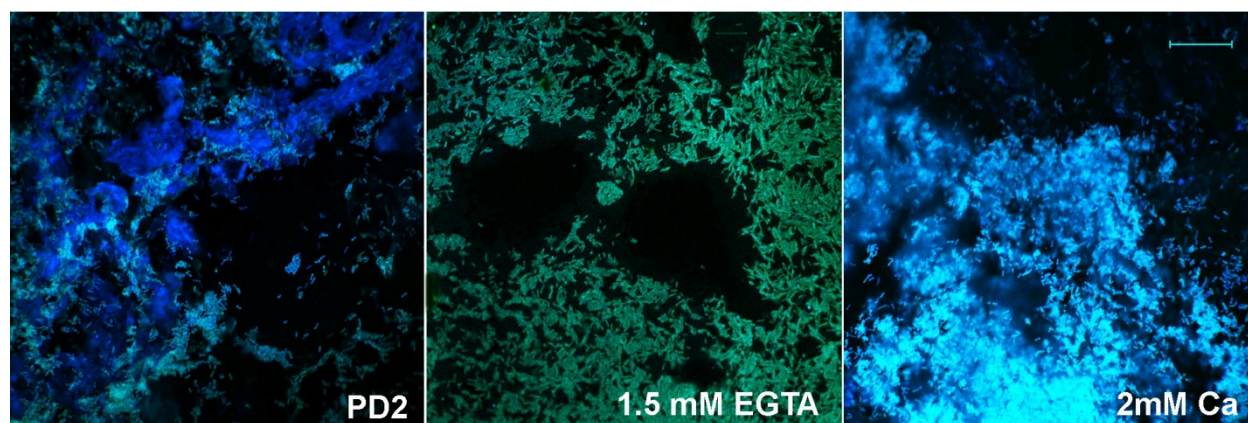


Figure 2-11. LSCM visualization of *X. fastidiosa* biofilm's exopolysaccharide.

PD2 supplemented with 2mM Ca or 1.5 mM EGTA immersed in batch cultures of an strain expressing green fluorescent protein (blue-green color in figures) grown in (A) PD2 or (B) PD2 supplemented with Ca. Two-week-old biofilms were stained with calcofluor white (darker bluish-white color in images) to indicate the presence of polysaccharides and observed by laser scanning confocal microscopy. Bar indicates 10 μ m.

Discussion

Two methods were used to assess differences in the production of EPS under the different Ca concentrations: calcofluor white, a visual indication of the amount of EPS, and expression analyses of two genes involved in the biosynthesis of the EPS, *gumD* and *gumH*, which incorporate glucose and mannose, respectively, to the EPS repetitive unit (da Silva et al., 2001). Both analyses corroborate the initial hypothesis that higher amount of EPS are present in biofilms formed under higher Ca concentrations. Quantification of total cell sugars by the phenol sulfuric acid method, included in this chapter, did not show significant differences in the amount of exopolysaccharide produced by biofilm cells under Ca-depleted conditions, non-supplemented media, and 2mM Ca supplemented media. This result may implicate low sensitivity of the previously used method to determine the differences found here. EPS production is a population-dependent trait (Scarpari et al., 2003), as we expected higher populations present in the biofilms of Ca treated cultures would produce more EPS/cell. The findings of this chapter indicted that Ca is playing a role in all the steps required in biofilm formation, attachment, aggregation, and production of EPS, which are known to be required for *X. fastidiosa* virulence.

References

- Clark, M.E., Edelman, R.E., Duley, M.L., Wall, J.D., and Fields, M.W. 2007. Biofilm formation in *Desulfovibrio vulgaris* Hildenborough is dependent upon protein filaments. *Environmental Microbiology* 9:2844-2854.
- da Silva, F.R., Vettore, A.L., Kemper, E.L., Leite, A., and Arruda, P. 2001. Fastidious gum: the *Xylella fastidiosa* exopolysaccharide possibly involved in bacterial pathogenicity. *Fems Microbiology Letters* 203:165-171.
- Livak, K.J., and Schmittgen, T.D. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2⁻($\Delta\Delta C_T$) method. *Methods* 25:402-408.
- Newman, K.L., R. P. P. Almeida, A. H. Purcell, and S. E. Lindow. 2003. Use of a green fluorescent strain for analysis of *Xylella fastidiosa* colonization of *Vitis vinifera*. *Appl Environ Microbiol* 69:7319-7327.
- Scarpari, L.M., Lambais, M.R., Silva, D.S., Carraro, D.M., and Carrer, H. 2003. Expression of putative pathogenicity-related genes in *Xylella fastidiosa* grown at low and high cell density conditions in vitro. *Fems Microbiology Letters* 222:83-92.
- Wood, P.J. 1980. Specificity in the interaction of direct dyes with polysaccharides. *Carbohydr Res* 85:271-287.
- Wood, P.J., Fulcher, R.G., and Stone, B.A. 1983. Studies on the specificity of interaction of cereal cell-wall components with Congo Red and Calcofluor - specific detection and histochemistry of (1- β 3),(1- β 4),-beta-D-glucan. *J Cereal Sci* 1:95-110.

Chapter 3 Calcium influence on twitching motility of *Xylella fastidiosa* involves regulation of one out of three *pilYI* homologues

Abstract

The plant pathogenic bacterium *Xylella fastidiosa* inhabits the xylem vessels of the host, where it forms biofilms thought to be responsible for disease symptoms. Twitching motility, dependent on type IV pili, is required for movement against the transpiration stream that results in basipetal migration and colonization. We previously demonstrated that increased concentrations of calcium result in increased *X. fastidiosa* motility. Further experiments were conducted to understand the molecular mechanisms of Ca-regulated twitching motility. Expression analyses of the type IV pili genes *pilA*, *pilT*, *pilB* and the three *pilYI* homologs (PD0023, PD0052, and PD1611) were performed on cells incubated in microfluidic chambers under replete and depleted Ca conditions. Up-regulation was indicated for *pilT*, encoding the retraction ATPase in charge of pili depolymerization, and one of the *pilYI* homologs (PD1611), encoding a protein presumably located at the tip of the pili. Sequence analysis identified a Ca-binding domain only in the up-regulated *pilYI*PD1611 homolog. Deletion mutagenesis of *pilYI*PD1611 results in reduced motility and a Ca-blinded phenotype, whereas twitching motility of a *pilYI*PD0023 mutant was still affected by Ca supplementation. Results indicate that the mechanism of Ca-dependent twitching motility is associated with Ca-binding by only one of the three *pilYI* homologs found in *X. fastidiosa*.

Introduction

Xylella fastidiosa is a Gram-negative, insect-vectorred plant-pathogenic bacterium that is the causal agent of a number of economically important diseases, most typically causing leaf scorch symptoms that are associated with bacterial xylem vessel colonization (Purcell, 1997). The development of diseases caused by *X. fastidiosa* is dependent on the ability of the bacteria to spread and become systemic within the xylem vessels, to which the bacterium is restricted (Hopkins and Purcell, 2002). Formation of bacterial biofilms is thought to produce disease symptoms (Hopkins, 1989). *X. fastidiosa* produces two types of fimbriae, type I pili (chaperone-usher type) (De La Fuente, 2013) and type IV pili, both located at one pole of the cell the surface (Meng et al., 2005). Both types of pili are involved in biofilm formation, cell attachment to the substrate and other cells, and motility. Particularly, the type I pilus is required primarily for cell anchoring, whereas the type IV pilus functions mainly as a facilitator of twitching motility and cell autoaggregation (Meng et al., 2005; De La Fuente et al., 2007b; De La Fuente et al., 2008). Twitching motility is characterized by extension, attachment to surfaces, and retraction of the type IV pili, pulling the cell forward (Mattick, 2002). *X. fastidiosa* twitching motility has been demonstrated in microfluidic chambers and *in planta*, where it determines cell movement against the transpiration stream for basipetal migration and long-distance colonization (Meng et al., 2005).

Besides motility, attachment, and biofilm formation, type IV pili mediate other functions in bacterial cells including competence and signaling (Mattick, 2002; Burdman et al., 2011). In many cases, mutations in the type IV pili result in a reduction or loss of pathogenicity (Shi and Sun, 2002; Burdman et al., 2011). Several genes involved in type IV pili biogenesis, function,

and regulation have been identified by mutagenesis of model organisms such as *P. aeruginosa* (Mattick, 2002; Burdman et al., 2011). The filamentous structure of type IV pili is composed of numerous units of a small protein called pilin encoded by *pilA*, which requires the processing of the peptidase /N-methylase PilD to become mature. Two ATPases, PilT and PilB, are in charge of assembly and disassembly of the polymeric structure for extension and retraction of the pilus, respectively, while the porin PilQ is implicated in their secretion (Mattick, 2002). Other genes involved in pilus response to environmental stimuli are the two-component sensor–regulator pair *pilR/pilS* (Winther-Larsen and Koomey, 2002) and the genes associated with a chemosensory regulatory system: *pilG–pilK* and *chpA–chpE* (Mattick, 2002).

In *X. fastidiosa*, various genes involved in type IV pili function and structure have been identified. Mutation of the type IV pili genes *pilQ*, *pilB*, *fimT*, *pilX*, and *pilO* prevented type IV pili production and motility in vitro (Meng et al., 2005; Li et al., 2007), while diminished motility was observed for a mutant in one of the *pilY1* homologs (PD0023) (De La Fuente et al., 2007a; Li et al., 2007). Additionally, different regulatory systems have been implicated in twitching modulation. For instance, the quorum sensing signaling molecule diffusible signal factor (DSF) represses motility as evidenced by a mutant in *rpffF*, the gene involved in synthesis of DSF, which spread faster in the plant vessels than the wild type (Chatterjee et al., 2008c). Other putative systems identified in the *X. fastidiosa* genome include the *pilR–pilS*, a two component regulatory system that activates transcription of *pilA* (Li et al., 2007), and the chemosensory Pil-Chp operon, with high homology to a chemosensory system in *P. aeruginosa*, which regulates type IV pili production and response (Cursino et al., 2011a).

Type IV pili and twitching motility have been observed in other Gram-negative bacteria including mammalian pathogens such as *Pseudomonas aeruginosa* (Burrows, 2012), *Neisseria meningitidis* (Albiger et al., 2003; Morand et al., 2004), *Neisseria gonorrhoeae* (Kallstrom et al., 1998; Wolfgang et al., 1998), and *Kingella kingae* (Porsch et al., 2013); and plant pathogens such as *Ralstonia solanacearum* (Liu et al., 2001), *Acidovorax citrulli* (Bahar et al., 2009), *Xanthomonas* spp. (Ojanen-Reuhs et al., 1997; Su et al., 1999; Wang et al., 2007; McCarthy et al., 2008), and *Pseudomonas* spp. (Suoniemi et al., 1995; Roine et al., 1998; Taguchi and Ichinose, 2011). Despite the knowledge gained on type IV pilus biology, the precise mechanism and factors triggering the pilus motor for its extension and retraction are still poorly understood.

In *Neisseria*, elongation and retraction of the pili was linked to PilC, a protein predicted to be at the tip of the type IV pili. Although dispensable for pili biogenesis, transcriptional regulation of *pilC* was directly correlated with the level of cell piliation (Morand et al., 2004). The model based on these findings proposed that up-regulation of *pilC* counteracts *pilT*, keeping the pilus extended by antagonizing its retractile action, while down regulation allows *pilT* function.

Posterior studies of *Pseudomonas syringae* and *K. kingae* established the presence of a stretch of nine amino acids at PilC/PilY1 C-terminus, corresponding to a Ca binding motif, which is also present in *Neisseria* (Orans et al., 2010; Porsch et al., 2013). Binding and release of Ca by the motive determines extension and retraction of the pili (Orans et al., 2010; Porsch et al., 2013).

Our research group previously reported that Ca increases twitching motility speed on agar plates as well as in microfluidic chambers (Cruz et al., 2012.Chapter 1). Because the *X. fastidiosa* environment, which consists of the xylem vessels, is in charge of transporting the plant mineral nutrients, it is expected that the presence and concentration of certain minerals may be used by

the bacterium as regulatory mechanisms. The present research was initiated to study the mechanism mediating Ca-dependent twitching motility in *X. fastidiosa*. In particular, we focus on the role of the three *pilYI* homologues, PD 0023, PD 0052 and PD 1611, present in this bacterium, and the conservation of this response in other plant-associated bacteria. Here we report the requirement of a 9- amino-acid Ca-binding motif for the regulation of the Ca-dependent twitching motility, which is present in one of *X. fastidiosa pilYI* homologs. As evidenced by a mutation of the Ca-binding containing *pilYI* homolog (PD 611) that results in Ca-unresponsive twitching phenotype, we demonstrate that PilY1 is a calcium dependent regulator of *X. fastidiosa* twitching motility

Materials and methods

Bacterial strains and culture conditions.

Xylella fastidiosa strain Temecula (ATCC 700964) was used as the wild-type (WT) strain in these experiments. Temecula mutants used in movement assessments are shown in Table 3-1. Bacterial strains with type IV pili assessed for twitching response to Ca and the media used for their evaluation are shown in Table 2. All *X. fastidiosa* strains were grown on PW solid media (Davis et al., 1980) or in PD2 liquid media (Davis et al., 1981b) at 28°C. Bacterial stocks were stored in PD2 broth with 20% glycerol at -80°C.

Table 3-1. *Xylella fastidiosa* strain Temecula mutants used in this study.

| Mutant | Locus Tag(s) | Features | Reference |
|--------------------------|--------------|------------------------------|--------------------------|
| <i>rpfF</i> | PD0407 | Defective in DSF production | Newman et al., 2004 |
| <i>rpfC</i> | PD0406 | Defective in DSF sensing DSF | Chatterjee et al., 2008b |
| <i>fimA</i> | PD0062 | Lacks type I pili | Meng et al., 2005 |
| <i>hxfA</i> | PD1792 | Lacks haemagglutinin A | Guilhabert et al., 2005 |
| <i>pilYI-0023</i> (TM14) | PD0023 | Lacks <i>pilYI</i> homolog | Li et al., 2007 |
| <i>pilYI-1611</i> | PD1611 | Lacks <i>pilYI</i> homolog | This work |

Movement quantification on agar plates and microfluidic chambers.

Twitching motility of the WT strain was assessed on plain PW containing BSA agar plates and PW agar plates separately supplemented with 0.25 mM CuSO₄, 2.0 mM MgSO₄, 1.5 mM FeSO₄, 1.0 mM ZnSO₄, 2 mM CaCl₂, 1.5 mM ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA), or 100μM 1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid, tetraacetoxymethyl esteris (BAPTA/AM). Twitching motility of *X. fastidiosa* mutant strains and other bacterial strains containing type IV pili (Table 3-2) was assessed in species-specific solid media (Table 3-2) containing 2 mM CaCl₂, 1.5 mM EGTA and non-supplemented media. Bacterial cells were spotted or stab inoculated in quadruplicate on triplicate agar plates and incubated at 28°C for five days for *X. fastidiosa* strains or two days for other bacterial species. Peripheral fringe width was quantified via microscopy at 40X phase-contrast optics on Eclipse Ti inverted microscope (Nikon, Melville, NY) using NIS-Elements version 3.01 software (Nikon). Three measurements were taken for each colony, for a total of 36 measurements per treatment.

Additionally, microfluidic chambers were used to assess the speed of WT in PD2 supplemented with 100 μm BAPTA/AM or 1.5 mM EGTA, the speed of *pilY1-1611* and *pilY1-0023* mutants in non-supplemented media or supplemented with 2 mM Ca, and the speed of *X. fastidiosa* (identified previously as mutant TM14 (Li et al., 2006). Experiments in the microfluidic chambers were repeated three times. The construction and design of the microfluidic chambers used for these experiments was as previously described (De La Fuente et al., 2007b). The microfluidic chambers were mounted onto the Eclipse Ti microscope and observed at 40X phase-contrast optics to monitor the cell speed. Cells were introduced into the chambers and

incubated for 2 h before time-lapse images were recorded with a Nikon DS-Q digital camera (Nikon, Melville, NY) every 30 s for 1 h. For the chelators' (BAPTA/AM and EGTA) experiments, cells were initially incubated in non-supplemented media. One day after inoculation, when twitching was observed, cells were treated with the chelators, and the speed of the cells was calculated every two hours during 14 hours. The movement of *X. fastidiosa* cells was quantified by tracking their positions frame by frame using NIS-Elements (Nikon, Melville, NY), and the cell speed was calculated by measuring the upstream displacement with respect to time.

Table 3-2 Bacterial strains with type IV pili used for assays of twitching motility response to Ca.

| Bacterial Species | Strain | Strain reference | Media* for twitching | Twitching reference |
|--|---------------|------------------------------|----------------------|----------------------------|
| <i>Acidovorax citrulli</i> | AAC00-1 | (Walcott et al., 2004) | NA | (Bahar et al., 2009) |
| <i>Pseudomonas fluorescens</i> | F113 | | LB, NA, SPA | (Martin et al., 1995) |
| <i>Pseudomonas syringae</i> pv. <i>tomato</i> | DC3000 | (Cuppels, 1986) | LB, NA, SPA | (Martin et al., 1995) |
| <i>Ralstonia pickettii</i> | JM-64 | (McInroy and Kloepper, 1995) | PW | N/A |
| <i>Ralstonia solanacearum</i> | GMI1000 | (Message et al., 1978) | BG | (Liu et al., 2001) |
| <i>Ralstonia solanacearum</i> | K60 | (Kelman, 1954) | BG | (Liu et al., 2001) |
| <i>Xanthomonas axonopodis</i> pv. <i>citrumelo</i> | F1 | (Cubero and Graham, 2002) | SB | (Kraiselburd et al., 2012) |
| <i>Xanthomonas campestris</i> pv. <i>vesicatoria</i> | 85-10 | (Canteros, 1990) | SB | (Kraiselburd et al., 2012) |
| <i>Xanthomonas albilineans</i> | FL07-1 GPE5SR | (Rott et al., 1996) | MW | |
| <i>Xylella fastidiosa</i> subsp. <i>fastidiosa</i> | Temecula | (Wells et al., 1987) | PW | (Meng et al., 2005) |

*Media abbreviations

BG = Bacto peptone-glucose agar (Liu et al., 2001)

LB = Luria-Bertani agar

NA = Nutrient agar

PW = Periwinkle wilt medium (Davis et al., 1980)

SB = Silva-Buddenhagen medium (Ou, 1985)

SPA= Sucrose-peptone agar (Fahy and Persley, 1983)

MW= Modified Wilbrink's medium (Rott et al., 2011)

Gene expression analysis.

The expression of the type IV pili genes *pilT*, *pilB*, *pilA*, and the three *pilYI* homologs (PD0023, PD0052, and PD1611) was analyzed in different settings (PW solid media, glass test tubes containing PD2, and microfluidic chambers) under replete and depleted Ca concentration (2 mM Ca or 1.5 mM EGTA, respectively). Eight-day old second passage plates of *X. fastidiosa* WT cells were used as the inoculum for all settings. Glass culture tubes containing 5 ml of liquid PD2 were inoculated with 10 µl cell suspension ($OD_{600} = 0.8$) and incubated with shaking (150 rpm) at 28°C for two days. Then, biofilm cells were scraped from the air-liquid interface. On the solid media plates, cells were spotted, incubated at 28°C, and collected after two days. In the microfluidic chambers, chambers were inoculated with a cell suspension, and cells were independently collected for each condition after two days, when cell twitching was observed, by increasing the speed of media flow until all cells were detached.

Total RNA from each treatment was extracted using Quick RNA MiniPrep (Zymo Research, Irvine, CA) according to the manufacturer's instructions. RNA was treated with DNase I (Thermo Scientific, Pittsburgh, PA) to remove contaminant DNA, and sample concentrations were determined with a NanoDrop 2000 spectrophotometer (Thermo scientific, Pittsburgh, PA). 200 µg of each sample was used to synthesize single-strand cDNA by reverse-transcription using

the Maxima First Strand cDNA Synthesis Kit for RT-qPCR (Thermo Scientific, Pittsburgh, PA). Equivalent amounts of cDNA were then used for quantitative PCR (qPCR). Primers for the type IV pili genes and the internal controls, *nuoA* and *gyrB*, were designed using PrimerQuest software (Integrated DNA Technologies) and are listed in Table 3. qPCRs (20 µl total volume) consisted of 1X Absolute Blue QPCR ROX mix (ABgene-Thermo Fisher Scientific, Surrey, United Kingdom), 0.4 µM 6-carboxyfluorescein-Black Hole quencher 1 (FAM-BHQ1)- labeled TaqMan probe, 0.2 µM each primer, and 1.5 µl of 200 ng/µl cDNA. Samples were amplified on an ABI 7500 real-time PCR system (Applied Biosystems, Foster City, CA), using the following cycling parameters: 95°C for 1 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Fold change in gene expression was calculated according to the $2^{-(\Delta\Delta CT)}$ method (Livak and Schmittgen, 2001). Data were analyzed using *nuoA* or *gyrB* endogenous controls to normalize samples. Changes in gene expression were assessed by t-test (fold change >1, $P < 0.05$) using Statistix v. 8.0 (Analytical Software, Tallahassee, FL).

In silico prediction and analysis of PilY1 Ca-binding motif.

Conserved domains in PilY1 were identified using the NCBI CD-search tool against the Pfam database. Amino acid sequences of PilY1 were retrieved from NCBI and analyzed with InterProScan to look for the presence of the Dx[DN]xDGxxD loop signature pattern of Ca-binding motif (Orans et al., 2010; Rigden et al., 2011). Secondary structure prediction was obtained with PSIPRED v3.3 (Jones, 1999; Buchan et al., 2010). Protein structure alignments were done using T-Coffee (Notredame et al., 2000).

Mutagenesis.

Homologous recombination was used to generate a *Xylella fastidiosa pilY1* (PD_1611) null mutant, *pilY1-1611*. First, *X. fastidiosa* WT genomic DNA was extracted using a modified cetyltrimethylammonium bromide (CTAB) protocol (Doyle and Doyle, 1987). Polymerase chain reaction (PCR) was then performed to amplify the 800 bp upstream and downstream fragments flanking the *pilY1* coding region. For this, primers P Up_F, P Up_R and pilY1 D_F, pilY1 D_R (Table 3) were designed using PrimerQuest software (Integrated DNA Technologies), and they contained AscI restriction site sequences added to the 5' end of the forward and reverse primers of the upstream and downstream segments. PCR reactions contained 1X buffer, 0.2 mM dNTPs, 0.2 μ M forward 0.2 μ M reverse primer, 0.625 U Taq polymerase, and 1 μ l of 50 ng/ μ l *X. fastidiosa* DNA in 25 μ l reactions. PCR was performed using the following parameters: 94°C for 1 min, followed by 49 cycles of 94°C for 30 sec and 70°C for 1 min, and a final extension step of 70°C for 10 min. The amplified segments were visualized by gel electrophoresis. PCR product was then digested with AscI, ligated with T4 ligase (Fisher, Pittsburgh, PA), and cloned into a pJET1.2/blunt cloning vector (Thermo Fisher Scientific, Pittsburgh, PA). The resulting plasmid, pJET_*pilY1*, was transformed into *Escherichia coli* NEB 5-alpha (New England BioLabs, Ipswich, MA). A kanamycin cassette was amplified from pUC4K, with primers containing the AscI restriction site sequence (Table3-3). pJET_*pilY1* and the kanamycin segment were digested with AscI and ligated with T4 ligase. To avoid re-ligation of the plasmid, pJET_*pilY1* was dephosphorylated with shrimp alkaline phosphatase (Affymetrix, USB, Santa Clara, CA) after restriction with AscI. The kanamycin segment and PJET_*pilY1* were ligated with T4 ligase. The final construct was electroporated into *E. coli* EAM1, a strain expressing a *X. fastidiosa*

methyltransferase (PD_1607) (Matsumoto and Igo, 2010) at the following conditions: 2.5 kV for 5.7 ms. Transformed EAM1 was incubated at 37°C with shaking at 200 rpm overnight in Luria-Bertani (LB) medium containing 1 mM IPTG and 50 mg ml⁻¹ kanamycin to select for resistance. Electrocompetent *X. fastidiosa* Temecula cells were prepared as previously described (Matsumoto et al., 2009). 50 µl of electrocompetent *X. fastidiosa* and 200 ng of methylated plasmid DNA were transferred to a 2 mm cuvette (Eppendorf, Hauppauge, NY) and electroporated at the following conditions: 2.5 kV for 5.7 ms. Immediately, 1 ml of PD3 medium (Davis et al., 1981a) was added to the transformed cells, which were incubated at 28°C with shaking at 150 rpm overnight. 200 µl of incubated cells were then spread-plated on PD3 solid media (solidified with Gelrite instead of agar) containing 30 µg ml⁻¹ of kanamycin. Plates were incubated at 28°C until transformants were visible (~10 days). DNA was extracted from resulting transformants using the previously mentioned CTAB protocol, PCR-amplified with primers designed from the regions flanking recombination region PY_DR and PilY1UP_F1 paired with primers designed from the kanamycin cassette Kan_F and PilY1UP_R1Kan (Table 3), and sequenced (Eurofins MWG Operon, Huntsville, AL) to confirm the mutation. Results were evaluated by BLAST (GenBank) to confirm the correct insertion of the kanamycin cassette and the absence of any sequence mismatches.

Transmission electron microscopy.

X. fastidiosa cells obtained from the peripheral fringe of 3-day-old bacterial cultures grown on PW agar plates were scraped off and suspended in 100 µl of 1X phosphate-buffered saline. 10 µl of the cell suspension was deposited on Formav-coated grids and allowed to settle for 1 min. The

cells were subsequently stained with 50 µl of phosphotungstic acid, the excess liquid was removed, and the grids were air dried and observed on a transmission electron microscope.

Table 3-3 PCR primers and probes used in this study

| Primer | Target gene | Sequence 5'-3' |
|--------------------------------------|-------------|----------------------------------|
| Mutagenesis | | |
| piY1D_F | | ACG GCGCGCC CACCGATGAACATGACGAAA |
| piY1D_R | | ACGGAATTCGTGAAGATTCGGCAGTGTGAGC |
| P Up-For | | ACGGAATTCGTTGTGTGCGGGTTTTGGT |
| P Up-Rev | | ACGGGCGCGCCGTGGGAACGTGTGAGCAAG |
| Kan Asc1_F | | TTAGGCGCGCCGTCTGCCTCGTGAAG |
| Kan Asc1_R | | AAAGGCGCGCCAAGCCACGTTGTGT |
| Mutation confirmation primers | | |
| PY_DR | | GGAGTGGAGTCCTTGCAATTT |
| Kan_F | | CACCTGATTGCCCGACATTA |
| PiY1UP_F1 | | GTGCAGCCTTGCCCTA |
| PiY1UP_R1Kan | | CACCAACTGGTCCACCTACAA |
| Gene expression analysis | | |
| piY1 1611_F | | GGTCGCATTGCAATATCCAGGCAA |
| pilY1 1611_R | | ACAGTAATGATGGGACGCGCAGTA |
| piY1 1611_probe | | AGTTTGTAGGGCTGTTGTCCTGCACA |
| piY1 0023_F | | TGATTGATGGTGGTGTCTG |
| piY1 0023_R | | CTACCCGTGTCCATTTGACC |
| piY1 0023_Probe | | GACAACCCTGATCCTGAGGA |
| piY1 0052_F | | ATTCCAAGCGTGTCCGCTAT |
| piY1 0052_R | | ATAGTACGACCCACCCGATGAGTA |
| piY1 0052_Probe | | CCCAATCCTGAGGATGCTAA |
| pilB_F | | AAGCTGGGTATCGACAAACTCGGT |
| pilB_R | | TCTTCGGCTGTGGAGATATTGCGT |
| pilB_Probe | | ACTGCGTTAGGCATCCTCAATGACGA |
| pilT_F | | CCGCAAACCTCATTGAAGAACCGCA |
| pilT_R | | CGCAGCCATGATCGACCATATCAACA |
| pilT_Probe | | AGCACTTCTGTGAGGTGTGAACGA |
| pilA_F | | AAACACCGGACTTGCCAACATCAC |
| pilA_R | | AAACCATCGCTTGGAATCGTAGCGTCGA |
| pilA_Probe | | TGTTGCATGTCCACTGACCTCCAT |
| gyrB_F | | TTGGCGTGATGGTTACCACTACCA |
| gyrB_R | | ATGTAGTACCGCGTTTCGCTGACA |
| gyrB_Probe | | TGGGTGAGCCGCAATATCCTCTTAAGCA |
| nuoA F | | AGACGCACGGATGAAGTTCGATGT |
| nuoA R | | ATTCCAGCGCTCCCTTCTTCATA |
| nuoA Probe | | TTCATCGTGCCTTGACTCAGGTGTT |

Results

Effect of metal supplementation and calcium chelation on the movement of *Xylella fastidiosa*.

Colony fringe measurements, an indicator of cell twitching movement, showed that, of all metals tested, only Ca supplementation produced a significant increase ($P \leq 0.001$) in *X. fastidiosa* WT movement compared with the non-supplemented PW plates. In contrast, a significant decrease in the fringe width was observed in colonies grown on media supplemented with Zn and Cu ($P \leq 0.001$). The fringes observed in Mg ($P = 0.12$) and Fe ($P = 0.80$) supplemented plates were not different from that observed in the non-supplemented plates (Fig. 3-1).

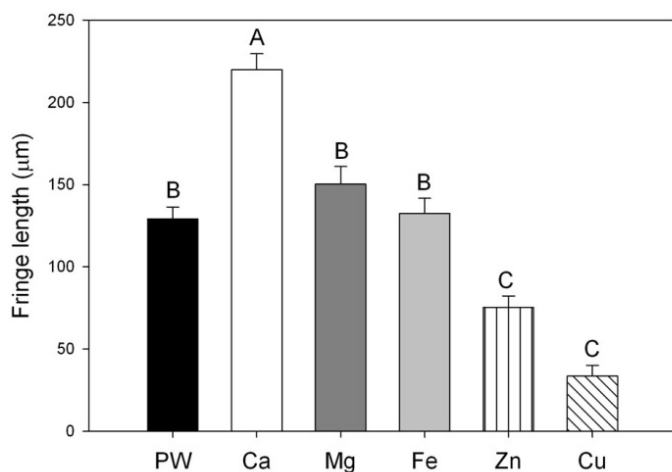


Figure 3-1. The effect of different metals on *Xylella fastidiosa* twitching motility.

Colony fringe width of *X. fastidiosa* WT spotted on PW agar and PW agar supplemented with 2 mM CaCl_2 , 2 mM MgSO_4 , 1.5 mM FeSO_4 , 1 mM ZnSO_4 , or 0.25 mM CuSO_4 . Different letters on the bars indicate statistically significant differences according to GLIMMIX procedure ($P \leq 0.05$). Error bars represent the standard error of the mean ($n=36$).

Compared with the non-supplemented PW plates, a small fringe was observed for colonies grown in media supplemented with the extracellular Ca chelator EGTA as previously reported (Cruz et al., 2012 Chapter 1). In contrast, colonies grown on plates containing the intracellular Ca chelator BAPTA/AM exhibited movement, as evidenced by their colony fringe, although it was significantly lower than that observed for non-supplemented PW or Ca-supplemented media ($P \leq 0.001$) (Fig. 3-2A and B). Analysis of the twitching motility of cells in the microfluidic chambers allowed comparison of treatments' effects on cell speed over time. Results of these experiments indicate movement of cells exposed to EGTA decreased more rapidly over time after exposure than for cells exposed to BAPTA/AM (Fig. 3-2C).

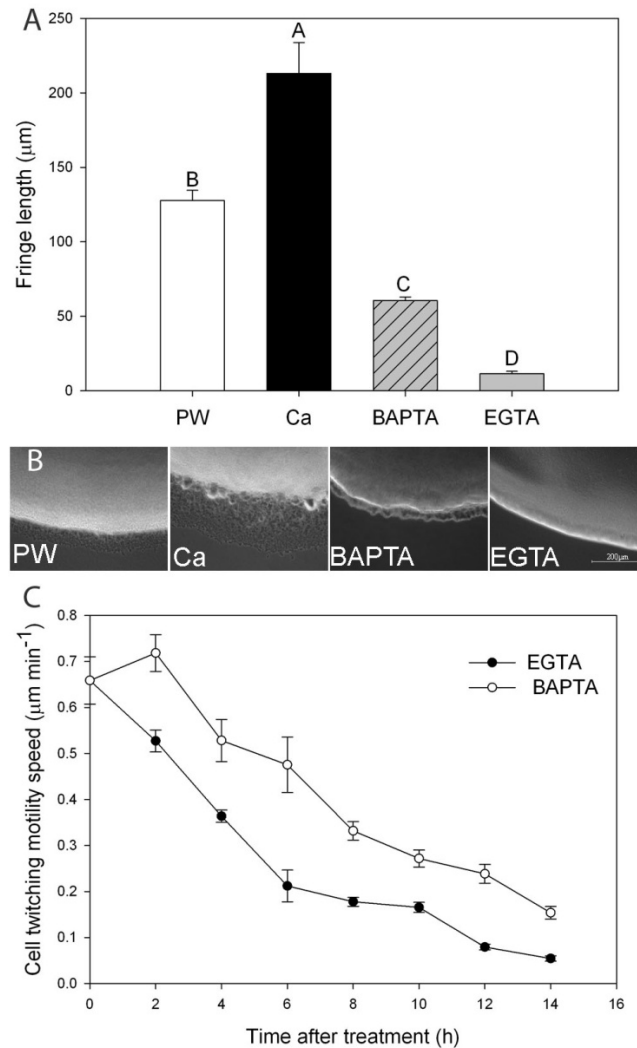


Figure 3-2. Effect of Ca and the extracellular and intracellular Ca chelators BAPTA and EGTA, respectively, on the twitching motility of the *X. fastidiosa* WT strain.

(A) Colony fringe width of *X. fastidiosa* cultured on PW agar plates and PW supplemented with 2 mM CaCl_2 , 1.5 mM EGTA, or 100 μM BAPTA. Different letters on the bars indicated statistically significant differences according to GLIMMIX procedure. Error bars represent standard error of the mean (n=36). (B) Light micrographs of the *X. fastidiosa* colonies on agar media with the treatments indicated above. (C) Twitching motility assessment in the microfluidic chambers after the addition of EGTA and BAPTA. The movement of the cells was recorded 14 h

after treatment addition. The speed of the cells was calculated for each two hour time lapse. Error bars at each point indicate the standard error of the mean (n=4).

Involvement of virulence genes in the twitching response to Ca.

To determine if biosynthetic genes implicated in twitching motility modulation (attenuation) are linked either directly (DSF) or indirectly (type I pili, hemmagglutinins) to the increase in cell twitching in response to Ca, *X. fastidiosa* mutants *fimA* (type I pili), *hxfA* (hemmagglutinins), *rpjF*, and *rpjC* (DSF) were assessed for movement on PW plates and PW plates supplemented with 1.5 mM EGTA or 2 mM Ca.

Twitching motility, evaluated by measuring the colony fringe, was significantly increased with the addition of Ca to the medium for the all the mutants evaluated (Figure 3): *fimA* ($P= 0.04$), *hxfA* ($P= 0.04$), *rpjF* ($P = \leq 0.001$), and *rpjC* ($P= 0.04$) (Fig 3-3). Additionally, a decrease or complete stop in movement was observed in EGTA-supplemented media compared to non-supplemented media for these mutants (data not shown).

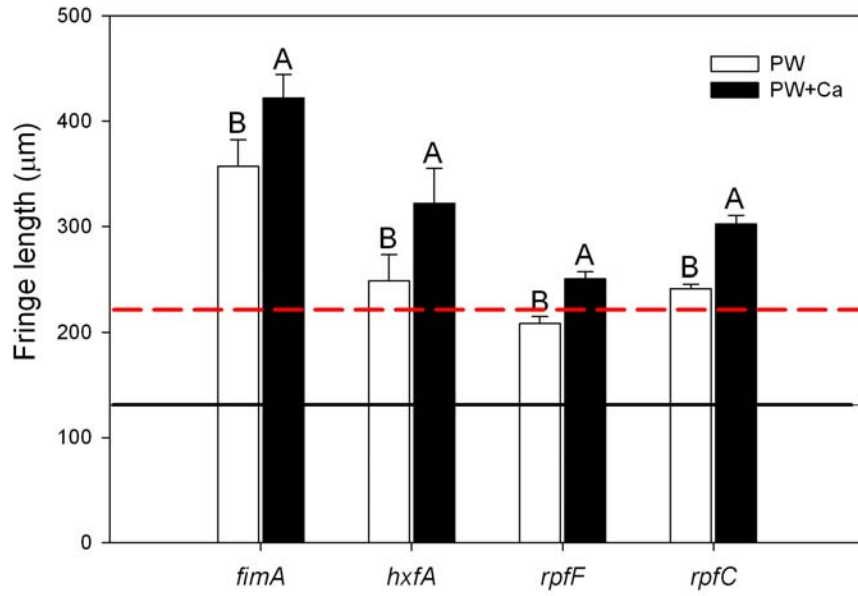


Figure 3-3. Effect of Ca on twitching motility of *X. fastidiosa* mutant strains defective in genes involved in the production of fimbriae (type I pili *fimA*), afimbrial adhesins (hemagglutinin *hxfA*), and DSF molecule biosynthesis (*rpfF*) and sensing (*rpfC*).

Twitching motility was quantified on PW plates and PW supplemented with 2 mM CaCl_2 . The horizontal line indicates WT fringe length on non-supplemented plates and, the dashed line indicates WT fringe supplemented with 2mM CaCl_2 . Different letters on the bars indicate significant differences according to the GLIMMIX procedure ($P \leq 0.05$). Error bars represent the standard errors of the means (n=36).

Expression of the type IV pili genes under replete and depleted Ca conditions.

Comparison of the expression of *X. fastidiosa* genes from bacteria incubated in media containing 2 mM Ca or 1.5 mM EGTA indicated no differences in expression for any of the genes evaluated (*pilT*, *pilB*, *pilA*, and the three *pilY1* homologs PD0023, PD0052, and PD1611) for cells collected

from glass test tubes as indicated by the t-test. Similarly, no differential expression was observed for genes isolated from cells incubated on plates containing 2 mM Ca or 1.5m M EGTA. In contrast, in the microfluidic chambers, a significant increase in gene expression in the 2mM CaCl₂-supplemented treatment compared to the EGTA treatment was detected for *pilT* (4.1 fold) and *pilY1-1611* (1.7 fold). A non-significant increase was observed for *pilB* (2.1 fold). As with culture tubes and plates, no change in expression was observed for the other two *pilY1* homologs (PD0023 and PD0052) in microfluidic chambers (Fig 3-4).

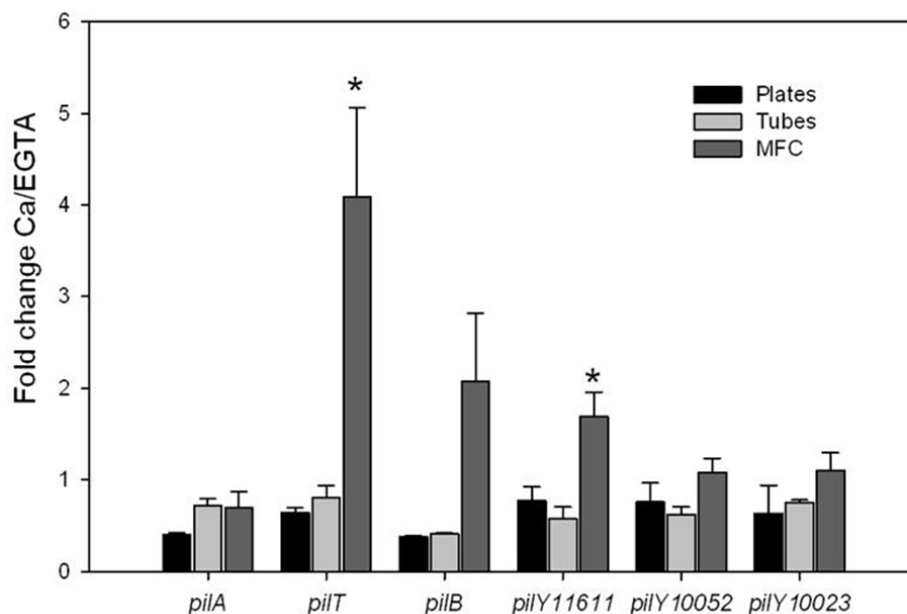


Figure 3-4. Gene expression analysis of type IV pili genes in three different settings: PW agar plates, glass tubes, and microfluidic chambers (MFC).

Results are expressed as the fold change in gene expression in the Ca treatment, considering EGTA as the basal condition. Asterisks indicate significant differences in gene expression as determined by the t-test (fold change >1, $P < 0.05$).

In silico analysis of PilY1 homologs for the presence of a conserved Ca-binding motif.

Conserved domain analysis of the amino acid sequences of the three PilY1 protein homologs present in *X. fastidiosa* revealed the presence of the *Neisseria* PilC beta-propeller domain in the C-terminal of the protein, spanning approximately 500 amino acids (Fig 3-5). This domain (pfam05567) has been identified in several PilC protein sequences from *N. gonorrhoeae* and *N. meningitidis*. The PilC protein associated with pilus-mediated adherence of pathogenic *Neisseria* spp. to target cells is homologous to PilY1. In addition, PilY1 homologs in *X. fastidiosa* were examined for the presence of the Ca-binding signature pattern Dx[DN]xDG as an indication the EF-hand-like calcium-binding site that has been shown to be conserved in other pathogens that produce type IV pili (Orans et al., 2010; Porsch et al., 2013). This analysis indicated that only one of the homologs exhibits the Ca-binding motif, a stretch of 9 amino acids (DTDGDGLVD) located between two β -strands according to secondary structure prediction. The structural context of this region agrees with what has been previously described for other PilY1 (PilC) proteins (Fig 3-5).

twitching motility when additional Ca was present in the media ($P \leq 0.001$). In contrast, experiments carried out with mutant *pilY1_1611* showed non-significant difference when PW or PD2 was supplemented with Ca in either agar plates ($P = 0.17$) or microfluidic chambers ($P = 0.55$).

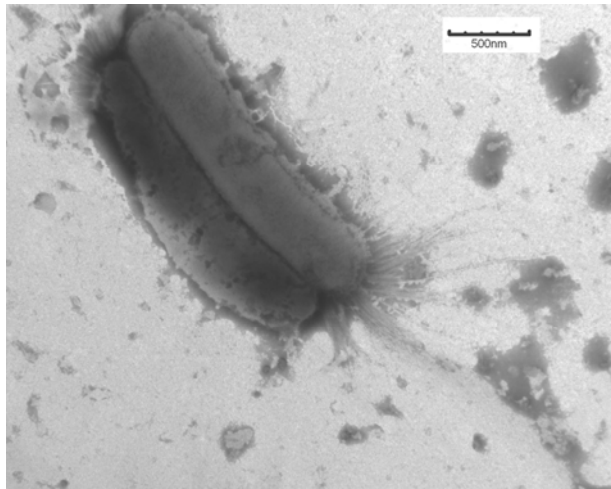


Figure 3-5. Transmission electron micrographs of negatively stained mutant *pilY1-1611* depicting both short type I pili and longer type IV pili.

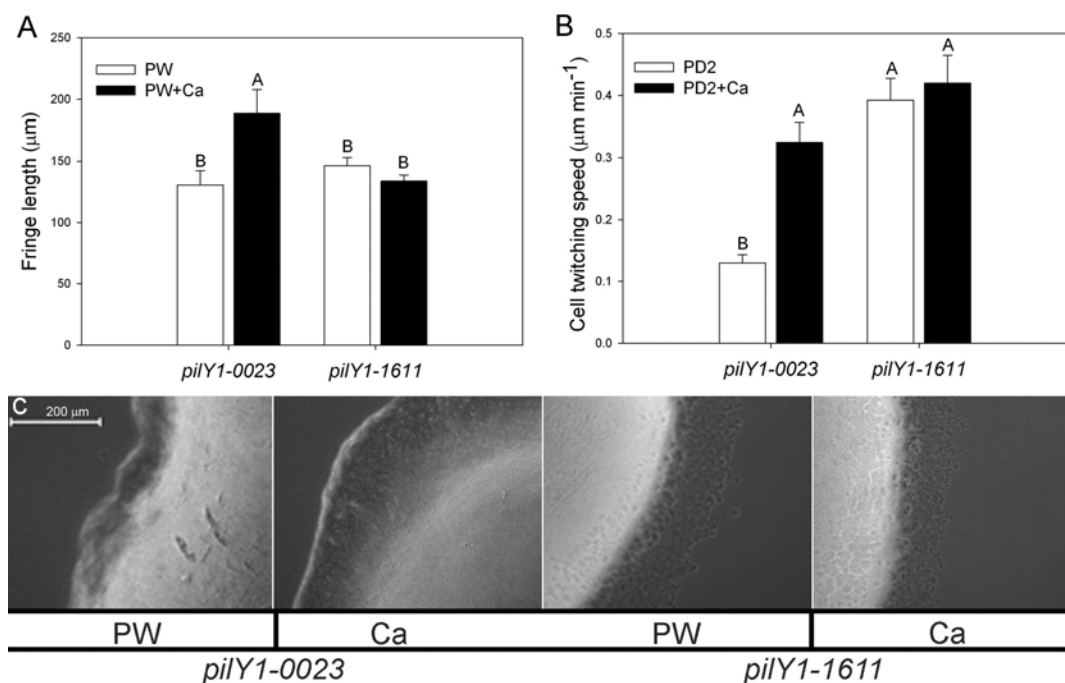


Figure 3-6. Twitching phenotypes of pilY1 mutant homologs.

A) Colony fringe width of *X. fastidiosa* mutant strains cultured on PW agar and PW supplemented with 2 mM CaCl₂. Different letters on the bars indicate statistically significant differences according to the GLIMMIX procedure ($P \leq 0.05$). Error bars represent standard errors of the mean ($n = 12$). B) Twitching speed assessments of cells incubated in PD2 or PD2 supplemented with 2 mM CaCl₂ in microfluidic chambers. Different letters on the bars indicate significant differences according to the GLIMMIX procedure ($P \leq 0.05$). Error bars represent standard errors of the mean ($n = 10$). C) Representative micrographs of colony fringe on agar plates in the treatments mentioned above

Effect of Ca on twitching motility of plant-associated bacteria

Examination of the peripheral morphology of the different bacterial strains incubated on plain media and media supplemented with 2 mM CaCl₂ indicated that only *Rastonia piketti* was able to

respond to an increase in Ca concentration (Figure 3-6). None of the other bacteria strains evaluated showed a response (Table 4). Sequence analysis of the multiple *pilYI* homologs amino-acid sequences identified in the bacterial species examined here indicated that only *Ralstonia piketti* contained the Dx[DN]xDGxxD patter corresponding to the Ca-binding (Rigden et al., 2011) motif in one of its homologs (Table 3-4).

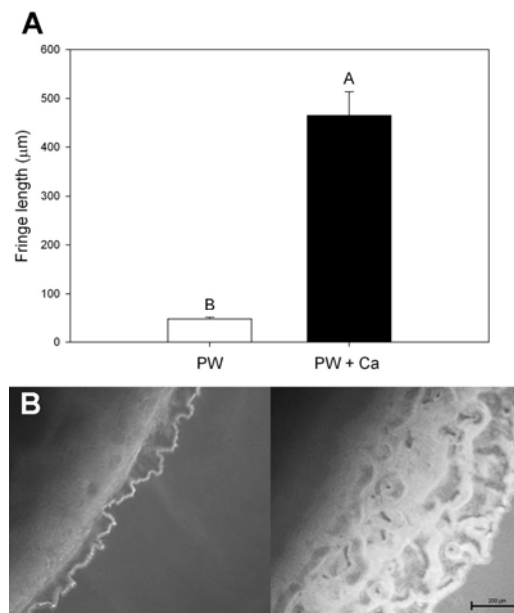


Figure 3-7 Effect of Ca on *Ralstonia piketti* twitching motility.

A) Colony fringe width of *R. piketti* cultured on PW agar plates and PW supplemented with 2 mM CaCl_2 . Different letters on the bars indicated statistically significant differences according to GLIMMIX procedure. Error bars represent standard error of the mean (n=24). B) Light micrographs of the colonies on agar media with the treatments indicated above.

Table 3-4. Presence of Ca-binding motif Dx[DN]xDG in PilY1 homologs of bacterial strains and the response of these strains to Ca.

Presence or absence of the motif is denoted as +/- . Response to Ca (+/-) is determined by an increase in twitching motility on solid media supplemented with Ca compared to the control in the present study unless otherwise specified.

| Bacterial Strain | Number of homologs | Ca-binding Motif | Response to Ca |
|--|---------------------------|-------------------------|-----------------------|
| <i>Acidovorax citrulli</i> AAC00-1 | 1 | - | - |
| <i>Pseudomonas fluorescens</i> F113 | 1 | - | - |
| <i>Pseudomonas syringae</i> pv. <i>tomato</i> DC3000 | 1 | - | - |
| <i>Ralstonia pickettii</i> JM-64 | 2 | + | + |
| <i>Ralstonia solanacearum</i> GMI1000 | 3 | - | - |
| <i>Ralstonia solanacearum</i> K60 | 2 | - | - |
| <i>Xanthomonas axonopodis</i> pv. <i>citrumelo</i> F1 | 1 | - | - |
| <i>Xanthomonas campestris</i> pv. <i>vesicatoria</i> 85-10 | 1 | - | - |
| <i>Xanthomonas albilineans</i> FLO7-1, GPE5SR | 1 | - | - |
| <i>Xylella fastidiosa</i> Temecula | 3 | + | + |

Discussion

As with other virulence traits, motility has been shown to be dependent on the presence and concentration of different metals in the environment that may act as signals inducing physiological changes. For instance, Fe has been linked with the modulation of twitching motility, which is associated with the production of the quorum sensing signal *N*-butanoyl homoserine lactone in *Pseudomonas aeruginosa* (Patriquin et al., 2008), and with the up-regulation of type IV pili genes in *X. fastidiosa* (Zaini et al., 2008). In *Vibrio fischeri*, motility and flagella production are dependent on Mg (O'Shea et al., 2006). In the present study, Ca is implicated in the mechanism of type IV pilus retraction in *Xylella fastidiosa*, as evidenced by the presence of a Ca-binding motif in the type IV pili protein PilY1 previously reported in PilY1 in *P. aeruginosa*, *Neisseria* spp., and *Kingella kingae*. This represents the first report of a Ca-binding motif located in PilY1 and involved in twitching motility in a plant pathogenic bacterium.

Twitching motility, dependent on the type IV pili, is a key virulence trait for many bacteria, including *X. fastidiosa*. Besides their function mediating motility, type IV pili can also be important in cell attachment for epiphytic or endophytic colonization, aggregation, biofilm formation, and other characteristics related to virulence, depending on the bacterium (Burdman et al., 2011). Research on twitching motility in *X. fastidiosa* shows that disruption of genes implicated in the production or function of the type IV pili result in delayed and less severe symptoms (Cursino et al., 2011b). Because of this, it is important to examine the potential effects of various metals on twitching motility to identify any which promote bacterial virulence by increasing twitching motility. Experiments examining the effects of media supplementation with

a suite of metals showed that, under in vitro experimental conditions, only Ca was able to increase twitching motility among the metals evaluated. It was hypothesized, then, that Ca plays an important role in the mechanism of twitching motility, likely interacting with type IV pili to promote twitching.

To determine the type of interaction with Ca causing the increase in twitching, the effects of intra- and extracellular chelation of Ca on bacterial phenotype were tested by measuring fringe length on agar plates. Chelation of extracellular Ca produced a larger decrease in twitching than intracellular chelation. However, the result for extracellular chelation could be affected by intracellular chelation as well. Colonies were incubated for four days, time during which several cell division cycles take place in a low Ca environment, when either an intra- or extracellular chelator is present. Thus, new cells should contain smaller amount of intracellular Ca, even when the intended removal is extracellular. Experiments in the microfluidic chambers, where chelators are introduced to the cells and then removed by media flow, reduced the impact of long exposure time to the chelators that also may affect other cellular processes that require Ca. Faster cell deceleration in microfluidic chambers observed in the extracellular chelation treatment (EGTA) confirmed plate result and indicates that Ca may interact with an extracellular structure to elicit the increase in motility. The reduction of motility by intracellular chelation, even if implicated in the Ca-dependent twitching response, may be partially explained by other essential roles of Ca in bacterial cell physiology.

In *X. fastidiosa*, attachment and twitching motility are oppositely regulated (Meng et al., 2005; Li et al., 2006). For instance, mutations in attachment or aggregation-associated proteins result in a

twitching-enhanced phenotype and accelerated appearance of symptoms in the host (Feil et al., 2007). Therefore, when assessing the effect of Ca on twitching motility, it is important to also assess any interference with bacterial attachment. Here, an indirect effect of Ca on the attachment and aggregation-associated proteins was tested by assessing the twitching speed of mutants for the hemagglutinin-encoding gene *hxfA* (Guilhabert and Kirkpatrick, 2005) and the type I pili encoding gene *fimA* (Meng et al., 2005) under different Ca conditions. The response to Ca was conserved in these mutants, suggesting that the effect of Ca enhancing twitching is not an indirect effect on the adhesion functions of these proteins (substrate attachment and cell-to-cell aggregation). Similarly quantification of twitching motility of DSF mutants *rpfF* and *rpfC* on agar plates supplemented with Ca in the present study indicate that the mechanism of Ca-mediated twitching motility is not associated with the production or sensing of DSF, as the increase in movement as a response to Ca supplementation was not altered.

To establish the effect of Ca on gene regulation among the type IV pili genes, gene expression was compared between Ca-supplemented and EGTA treated cells in test tubes, agar plates, and microfluidic chambers. Significant upregulation of *pilT*, and one *pilYI* homolog (PD1611) in Ca-supplemented media was only detectable in microfluidic chambers, indicating this is probably the most conducive environment for twitching motility. The other settings tested did not show differences in the expression; this is likely due to a high noise/signal ratio. In agar plates, the entire colony were used for expression analyses, but actively twitching cells may be only located at the fringe of the colony. In test tubes cultures, biofilm cells were used for expression analyses, but it is unknown what percentage of these cells might be actively twitching.

The amino acid sequence of the upregulated PilY1 homolog of *X. fastidiosa* contains a Ca-binding motif in its C-terminal portion. In *P. aeruginosa*, the proposed mechanism of Ca-dependent associates Ca binding a release by the protein to the extension and retraction of the type IV pili (Orans et al., 2010) In *Neisseria* spp. expression analysis demonstrated that extension and retraction of type IV pili was determined by the alternate expression of *pilT* and a *pilY1* homolog annotated as *pilC* in *Neisseria* spp (Monrand et al 2004). The experimental system used for the researchers allowed to separately evaluated cells exhibiting either extended or retracted pilus (Morand et al., 2004). Expression analyses in this work, analyzed a mixed population of cells in both states and, indicated upregulation of *pilT*, *pilY1*, under Ca-supplementation compared with Ca depleted conditions that may be in agreement with this model of Ca regulation. The exact effect of Ca on PilY1 is unknown; however Ca is associated with protein folding and conformational changes (Reid et al., 1981), that may be responsible for a changes in protein structure necessary for twitching, as has been speculated (Porsch et al., 2013).

In contrast to *P. aeruginosa*, which encodes a single PilY1 protein, *Neisseria* spp. and *K. kingae*, encode two PilY1 homologs annotated PilC1 and PilC2, but only PilC1 contains the Ca-binding motif (Mornad et al., 2004; Porche et al., 2013). In these two pathogens, the PilY1 containing the Ca-binding motif is dispensable for the production of the pili, but they regulate the level of cell piliation and the mechanism of twitching and cell attachment (Morand et al., 2004). Here, assessments of *X. fastidiosa* mutants for two of the PilY1 homologs (PD 1611 and PD 0023) indicated that neither is required for pilus biogenesis but both mutations decrease twitching motility, indicating that they may affect the level of piliation or the function of the type IV pili.

In *Kingella* and *Neisseria* spp., only when both homologs are mutated there is no production of pili (Morand et al., 2009; Porsch et al., 2013). In order to determine if a similar function in pilus biogenesis is accomplished by the *pilYI* homologs in *X. fastidiosa* a simultaneous mutation of the three homologs would be required. In addition, mutation of *pilYI* PD 1611, the Ca-binding containing homolog in *X. fastidiosa*, resulted in a Ca-blinded phenotype, indicating this homolog is involved in the response to Ca. In contrast, the *pilYI* PD0023 mutant, lacking the Ca-binding motif, maintains responsiveness to Ca.

Additional evidence of the involvement of this motif in twitching regulation was established by testing the twitching phenotype of different bacterial strains encoding type IV pili under Ca supplementation. These experiments showed a correlation between increase in motility and the presence of the Ca-binding motif in the PilY1 sequence of the soil inhabitant *Ralstonia piketti*. The presence of this motif should be directly related to the occurrence and concentration of Ca in niche of these bacteria, making it regulatory factor. *R. piketti* has been isolated from soil, water and plants (Coenye et al., 2003). Reports indicated its ability to survive and thrive in low nutrient (oligotrophic) conditions (McAlister et al., 2002), similarly *X. fastidiosa* lives in a nutrient dilute-environment, the xylem sap. Other reports indicated that, *R. piketti* is found as part of the human commensal microflora of the oral cavity and upper respiratory tract. However, it has been shown to be involved in a number of clinical infections (Stelzmueller et al., 2006). Under special conditions in immunodepressed individuals it becomes a pathogen able to infect the respiratory track and the blood stream (Coenye et al., 2002). Likely its ability as a human pathogen and the presence of specific concentrations of Ca in this environment is what explains the requirement of the Ca-binding motif for the regulation of the type IV pili twitching and attachment, as has been observed in other human pathogens with a PilY1 containing the same motif.

Based on the data presented in this work, it is hypothesized that the mechanism governing Ca-dependent twitching motility in *X. fastidiosa* is similar to what is described for *N. meningitidis* and *P. aeruginosa*, where Ca binding by the PilY1 elicits pilus polymerization for extension, and release allows mediated depolymerization for retraction. In addition, features specific to *X. fastidiosa* regulation of twitching were revealed. Individual mutation of the two *pilY1* genes studied here decreases but does not abolish twitching, indicating that may have a complementary function in twitching and that the mechanism of Ca-regulation is not indispensable for twitching but required for its regulation under specific conditions. Thus, it is possible to think that a prerequisite of Ca-dependent regulation of twitching in *X. fastidiosa* is the expression of the type IV genes and the production of the type IV pilus that enables the sensing of environmental Ca. Previous work by this group demonstrated that Ca increases cell attachment, cell autoaggregation, and biofilm formation in addition to the increasing twitching motility (Cruz et al., 2012 Chapter 1). How Ca does regulate two opposite phenotypes in *X. fastidiosa*? Because the phenotypes of *X. fastidiosa* vary in different phases of colonization, this process would require eventually either cell motility or biofilm formation, and this is dependent on the quorum-sensing mechanism. Although we found here that producing or sensing the quorum sensing molecule DSF does not affect the twitching response to Ca, further studies will be useful in understanding the role of Ca and quorum sensing in biofilm. More importantly, it will be interesting to study this problem inside *X. fastidiosa*' hosts, where other environmental cues are in play.

Acknowledgments

This project was supported by Agriculture and Food Research Initiative Competitive Grants Program grant no. 2010-65108-20633 from the USDA National Institute of Food and Agriculture and the Alabama Agricultural Experiment Station (AAES) Hatch Grant Program.

We thank Harvey C. Hoch and Thomas J. Burr (Cornell University) for *X. fastidiosa* mutant strains Michael Igo (University of California Davis) for constructs and strain for mutagenesis; Nian Wang (University of Florida) for *Xanthomonas* spp. strains; Ron Walcott (University of Georgia) for *Acidovorax citrulli*; Joseph Kloepper (Auburn University) for *Ralstonia pickettii* and *Pseudomonas syringae* strains; Timothy P. Denny (University of Georgia) for *Ralstonia solanacearum* strains; Dean Gabriel (University of Florida) for *Xanthomonas albilineans* strains; Fergal O’Gara (University College Cork) for *Pseudomonas fluorescence* strains. We acknowledge the Auburn University Confocal Facilities for the use of their microscope and Michael E. Miller for assistance with its operation.

References

- Albiger, B., Johansson, L., and Jonsson, A.B. 2003. Lipooligosaccharide-deficient *Neisseria meningitidis* shows altered pilus-associated characteristics. *Infection and Immunity* 71:155-162.
- Bahar, O., Goffer, T., and Burdman, S. 2009. Type IV pili are required for virulence, twitching motility, and biofilm formation of *Acidovorax avenae* subsp. *citrulli*. *Molecular Plant-Microbe Interactions* 22:909-920.
- Buchan, D.W.A., Ward, S.M., Lobley, A.E., Nugent, T.C.O., Bryson, K., and Jones, D.T. 2010. Protein annotation and modelling servers at University College London. *Nucleic Acids Research* 38:W563-W568.
- Burdman, S., Bahar, O., Parker, J.K., and De La Fuente, L. 2011. Involvement of type IV pili in pathogenicity of plant pathogenic bacteria. *Genes* 2:706-735.
- Burrows, L.L. 2012. *Pseudomonas aeruginosa* twitching motility: type IV pili in action. *Annual Review of Microbiology*, Vol 66 66:493-520.
- Canteros, B.J. (1990). Diversity of plasmids and plasmid-encoded phenotypic traits in *Xanthomonas campestris* pv. *vesicatoria* (Gainesville, Florida: University of Florida).
- Chatterjee, S., Almeida, R.P., and Lindow, S. 2008a. Living in two worlds: the plant and insect lifestyles of *Xylella fastidiosa*. *Annu Rev Phytopathol* 46:243-271.
- Chatterjee, S., Wistrom, C., and Lindow, S.E. 2008b. A cell-cell signaling sensor is required for virulence and insect transmission of *Xylella fastidiosa*. *Proceedings of the National Academy of Sciences* 105:2670-2675.
- Chatterjee, S., Newman, K.L., and Lindow, S.E. 2008c. Cell-to-cell signaling in *Xylella fastidiosa* suppresses movement and xylem vessel colonization in grape. *Mol. Plant Microbe Interact.* 21:1309-1315.
- Coenye, T., Vandamme, P., and LiPuma, J.J. 2002. Infection by *Ralstonia* species in cystic fibrosis patients: identification of *R. pickettii* and *R. mannitolilytica* by polymerase chain reaction. *Emerging infectious diseases* 8:692-696.
- Coenye, T., Goris, J., De Vos, P., Vandamme, P., and LiPuma, J.J. 2003. Classification of *Ralstonia pickettii*-like isolates from the environment and clinical samples as *Ralstonia insidiosa* sp. nov. *International journal of systematic and evolutionary microbiology* 53:1075-1080.
- Cruz, L.F., Cobine, P.A., and De La Fuente, L. 2012. Calcium increases *Xylella fastidiosa* surface attachment, biofilm formation, and twitching motility. *Appl Environ Microbiol* 78:1321-1331.
- Cubero, J., and Graham, J.H. 2002. Genetic relationship among worldwide strains of *Xanthomonas* causing canker in citrus species and design of new primers for their identification by PCR. *Appl Environ Microbiol* 68:1257-1264.
- Cuppels, D.A. 1986. Generation and characterization of Tn5 insertion mutations in *Pseudomonas syringae* pv. *tomato*. *Appl Environ Microbiol* 51:323-327.
- Cursino, L., Galvani, C.D., Athinuwat, D., Zaini, P.A., Li, Y.X., De La Fuente, L., Hoch, H.C., Burr, T.J., and Mowery, P. 2011a. Identification of an operon, Pil-Chp, that controls twitching motility and virulence in *Xylella fastidiosa*. *Molecular Plant-Microbe Interactions* 24:1198-1206.

- Cursino, L., Galvani, C.D., Athinuwat, D., Zaini, P.A., Li, Y., De La Fuente, L., Hoch, H.C., Burr, T.J., and Mowery, P. 2011b. Identification of an operon, Pil-Chp, that controls twitching motility and virulence in *Xylella fastidiosa*. *Molecular plant-microbe interactions* : MPMI 24:1198-1206.
- Davis, M.J., Purcell, A.H., and Thomson, S.V. 1980. Isolation media for the Pierce's disease bacterium. *Phytopathology* 70:425-429.
- Davis, M.J., Whitcomb, R.F., and Gillaspie, A.G.J. 1981a. Fastidious bacteria of plant vascular tissue and invertebrates (including so called rickettsia-like bacteria). Pages 2172-2188 in: *The prokaryotes : a handbook on habitats, isolation, and identification of bacteria*, M.P. Starr, H. Stolp, H.G. Truper, A. Balows, and H.G. Schlegel, eds. Springer-Verlag, Berlin ; New York.
- Davis, M.J., French, W.J., and Schaad, N.W. 1981b. Axenic culture of the bacteria associated with phony disease of peach and plum leaf scald. *Current Microbiology* 6:309-314.
- De La Fuente, L., Burr, T.J., and Hoch, H.C. 2007a. Mutations in type I and type IV pilus biosynthetic genes affect twitching motility rates in *Xylella fastidiosa*. *J Bacteriol* 189:7507-7510.
- De La Fuente, L., Burr, T.J., and Hoch, H.C. 2008. Autoaggregation of *Xylella fastidiosa* cells is influenced by type I and type IV pili. *Appl. Environ. Microbiol.* 74:5579-5582.
- De La Fuente, L., Galvani, C.D., Cursino, L., Burr, T.J., and Hoch, H.C. 2007b. *Xylella fastidiosa* movement and biofilm formation studied in artificial xylem vessels. *Phytopathology* 97:S26-S26.
- De La Fuente, L., M. Igo, P. Zaini, J.K. Parker, and S. Burdman 2013. Fimbrial and afimbrial proteins involved in bacterial attachment. in: *Virulence mechanisms of plant pathogenic bacteria*, American Phytopathological Society Press, in press.
- Doyle, J., and Doyle, J.L. 1987. Genomic plant DNA preparation from fresh tissue - CTAB method. *Phytochem. Bull.* 19:11-15.
- Fahy, P.C., and Persley, G.J. 1983. *Plant bacterial diseases: a diagnostic guide*. Academic Press, New York.
- Feil, H., Feil, W.S., and Lindow, S.E. 2007. Contribution of fimbrial and afimbrial adhesins of *Xylella fastidiosa* to attachment to surfaces and virulence to grape. *Phytopathology* 97:318-324.
- Guilhabert, M.R., and Kirkpatrick, B.C. 2005. Identification of *Xylella fastidiosa* antivirulence genes: hemagglutinin adhesins contribute to *Xylella fastidiosa* biofilm maturation and colonization and attenuate virulence. *Molecular Plant-Microbe Interactions* 18:856-868.
- Hopkins, D.L. 1989. *Xylella fastidiosa* - xylem-limited bacterial pathogen of plants. *Annual Review of Phytopathology* 27:271-290.
- Hopkins, D.L., and Purcell, A.H. 2002. *Xylella fastidiosa*: cause of Pierce's disease of grapevine and other emergent diseases. *Plant Disease* 86:1056-1066.
- Jones, D.T. 1999. Protein secondary structure prediction based on position-specific scoring matrices. *Journal of Molecular Biology* 292:195-202.
- Kallstrom, H., Islam, M.S., Berggren, P.O., and Jonsson, A.B. 1998. Cell signaling by the type IV pili of pathogenic *Neisseria*. *Journal of Biological Chemistry* 273:21777-21782.
- Kelman, A. 1954. The relationship of pathogenicity in *Pseudomonas solanacearum* to colony appearance on a tetrazolium medium. *Phytopathology* 44:693-695.

- Kraiselburd, I., Alet, A.I., Tondo, M.L., Petrocelli, S., Daurelio, L.D., Monzon, J., Ruiz, O.A., Losi, A., and Orellano, E.G. 2012. A LOV protein modulates the physiological attributes of *Xanthomonas axonopodis* pv. *citri* relevant for host plant colonization. Plos One 7.
- Li, Y., Hao, G., Galvani, C.D., Meng, Y., De La Fuente, L., Hoch, H.C., and Burr, T.J. 2007. Type I and type IV pili of *Xylella fastidiosa* affect twitching motility, biofilm formation and cell-cell aggregation. Microbiology 153:719-726.
- Li, Y.X., Hao, G.X., Meng, Y.Z., Galvani, C.D., Hoch, H.C., and Burr, T.J. 2006. Genes required for twitching motility in *Xylella fastidiosa*. Phytopathology 96:S180-S180.
- Liu, H.L., Kang, Y.W., Genin, S., Schell, M.A., and Denny, T.P. 2001. Twitching motility of *Ralstonia solanacearum* requires a type IV pilus system. Microbiology-Sgm 147:3215-3229.
- Livak, K.J., and Schmittgen, T.D. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25:402-408.
- Martin, P.R., Watson, A.A., Mccaul, T.F., and Mattick, J.S. 1995. Characterization of a five-gene cluster required for the biogenesis of type 4 fimbriae in *Pseudomonas aeruginosa*. Molecular Microbiology 16:497-508.
- Matsumoto, A., and Igo, M.M. 2010. Species-specific type II restriction-modification system of *Xylella fastidiosa* Temecula1. Appl Environ Microbiol 76:4092-4095.
- Matsumoto, A., Young, G.M., and Igo, M.M. 2009. Chromosome-based genetic complementation system for *Xylella fastidiosa*. Appl Environ Microbiol 75:1679-1687.
- Mattick, J.S. 2002. Type IV pili and twitching motility. Annu Rev Microbiol 56:289-314.
- McAlister, M.B., Kulakov, L.A., O'Hanlon, J.F., Larkin, M.J., and Ogden, K.L. 2002. Survival and nutritional requirements of three bacteria isolated from ultrapure water. Journal of Industrial Microbiology & Biotechnology 29:75.
- McCarthy, Y., Ryan, R.P., O'Donovan, K., He, Y.Q., Jiang, B.L., Feng, J.X., Tang, J.L., and Dow, J.M. 2008. The role of PilZ domain proteins in the virulence of *Xanthomonas campestris* pv. *campestris*. Mol Plant Pathol 9:819-824.
- McInroy, J.A., and Kloepper, J.W. 1995. Survey of indigenous bacterial endophytes from cotton and sweet corn. Plant Soil 173:337-342.
- Meng, Y.Z., Li, Y.X., Galvani, C.D., Hao, G.X., Turner, J.N., Burr, T.J., and Hoch, H.C. 2005. Upstream migration of *Xylella fastidiosa* via pilus-driven twitching motility. Journal of Bacteriology 187:5560-5567.
- Message, B., Boistard, P., Pitrat, M., Schmit, J., and Boucher, C. 1978. A new class of fluidal avirulent mutant of *Pseudomonas solanacearum* unable to induce a hypersensitive reaction. Pages 823-833 in: Proceedings of the IVth International Conference on Plant Pathogenic Bacteria., Institut National de la Recherche Agronomique, Angers, France.
- Morand, P.C., Drab, M., Rajalingam, K., Nassif, X., and Meyer, T.F. 2009. *Neisseria meningitidis* differentially controls host cell motility through PilC1 and PilC2 components of type IV pili. Plos One 4.
- Morand, P.C., Bille, E., Morelle, S., Eugene, E., Beretti, J.L., Wolfgang, M., Meyer, T.F., Koomey, M., and Nassif, X. 2004. Type IV pilus retraction in pathogenic *Neisseria* is regulated by the PilC proteins. Embo J. 23:2009-2017.
- Notredame, C., Higgins, D.G., and Heringa, J. 2000. T-Coffee: A novel method for fast and accurate multiple sequence alignment. Journal of Molecular Biology 302:205-217.

- O'Shea, T.M., Klein, A.H., Geszvain, K., Wolfe, A.J., and Visick, K.L. 2006. Diguanylate cyclases control magnesium-dependent motility of *Vibrio fischeri*. *Journal of Bacteriology* 188:8196-8205.
- Ojanen-Reuhs, T., Kalkkinen, N., Westerlund-Wikstrom, B., van Doorn, J., Haahtela, K., Nurmiaho-Lassila, E.L., Wengelnik, K., Bonas, U., and Korhonen, T.K. 1997. Characterization of the *fimA* gene encoding bundle-forming fimbriae of the plant pathogen *Xanthomonas campestris* pv. *vesicatoria*. *J Bacteriol* 179:1280-1290.
- Orans, J., Johnson, M.D.L., Coggan, K.A., Sperlazza, J.R., Heiniger, R.W., Wolfgang, M.C., and Redinbo, M.R. 2010. Crystal structure analysis reveals *Pseudomonas* PilY1 as an essential calcium-dependent regulator of bacterial surface motility. *Proceedings of the National Academy of Sciences of the United States of America* 107:1065-1070.
- Ou, S.H. 1985. Part II: Bacterial diseases. Pages 61-108 in: *Rice diseases*, S.H. Ou, ed. Commonwealth Mycological Institute, Kew, Surrey, UK.
- Patriquin, G.M., Banin, E., Gilmour, C., Tuchman, R., Greenberg, E.P., and Poole, K. 2008. Influence of quorum sensing and iron on twitching motility and biofilm formation in *Pseudomonas aeruginosa*. *Journal of Bacteriology* 190:662-671.
- Porsch, E.A., Johnson, M.D., Broadnax, A.D., Garrett, C.K., Redinbo, M.R., and St Geme, J.W., 3rd. 2013. Calcium binding properties of the *Kingella kingae* PilC1 and PilC2 proteins have differential effects on type IV pilus-mediated adherence and twitching motility. *J Bacteriol* 195:886-895.
- Rigden, D.J., Woodhead, D.D., Wong, P.W.H., and Galperin, M.Y. 2011. New structural and functional contexts of the Dx[DN]xDG linear motif: insights into evolution of calcium-binding proteins. *Plos One* 6.
- Roine, E., Raineri, D.M., Romantschuk, M., Wilson, M., and Nunn, D.N. 1998. Characterization of type IV pilus genes in *Pseudomonas syringae* pv. *tomato* DC3000. *Molecular Plant-Microbe Interactions* 11:1048-1056.
- Rott, P., Fleites, L., Marlow, G., Royer, M., and Gabriel, D.W. 2011. Identification of new candidate pathogenicity factors in the xylem-invading pathogen *Xanthomonas albilineans* by transposon mutagenesis. *Molecular plant-microbe interactions : MPMI* 24:594-605.
- Rott, P.C., Costet, L., Davis, M.J., Frutos, R., and Gabriel, D.W. 1996. At least two separate gene clusters are involved in albicidin production by *Xanthomonas albilineans*. *J Bacteriol* 178:4590-4596.
- Shi, W.Y., and Sun, H. 2002. Type IV pilus-dependent motility and its possible role in bacterial pathogenesis. *Infection and Immunity* 70:1-4.
- Stelzmueller, I., Biebl, M., Wiesmayr, S., Eller, M., Hoeller, E., Fille, M., Weiss, G., Lass-Floerl, C., and Bonatti, H. 2006. *Ralstonia pickettii*-innocent bystander or a potential threat? *Clinical microbiology and infection : the official publication of the European Society of Clinical Microbiology and Infectious Diseases* 12:99-101.
- Su, W.C., Tung, S.Y., Yang, M.K., and Kuo, T.T. 1999. The *pilA* gene of *Xanthomonas campestris* pv. *citri* is required for infection by the filamentous phage cf. *Mol Gen Genet* 262:22-26.
- Suoniemi, A., Bjorklof, K., Haahtela, K., and Romantschuk, M. 1995. Pili of *Pseudomonas syringae* pathovar *syringae* enhance initiation of bacterial epiphytic colonization of bean. *Microbiol-Uk* 141:497-503.

- Taguchi, F., and Ichinose, Y. 2011. Role of type IV pili in virulence of *Pseudomonas syringae* pv. *tabaci* 6605: correlation of motility, multidrug resistance, and HR-inducing activity on a nonhost plant. *Molecular plant-microbe interactions* : MPMI 24:1001-1011.
- Walcott, R.R., Fessehaie, A., and Castro, A.C. 2004. Differences in pathogenicity between two genetically distinct groups of *Acidovorax avenae* subsp. *citrulli* on cucurbit hosts. *J Phytopathol* 152:277-285.
- Wang, L., Makino, S., Subedee, A., and Bogdanove, A.J. 2007. Novel candidate virulence factors in rice pathogen *Xanthomonas oryzae* pv. *oryzicola* as revealed by mutational analysis. *Appl Environ Microbiol* 73:8023-8027.
- Wang, N., Li, J.L., and Lindow, S.E. 2012. RpfF-dependent regulon of *Xylella fastidiosa*. *Phytopathology* 102:1045-1053.
- Wells, J.M., Raju, B.C., Hung, H.Y., Weisburg, W.G., Mandelcopaul, L., and Brenner, D.J. 1987. *Xylella fastidiosa* gen. nov., sp. nov: Gram-negative, xylem-limited, fastidious plant bacteria related to *Xanthomonas* spp. *Int J Syst Bacteriol* 37:136-143.
- Winther-Larsen, H.C., and Koomey, M. 2002. Transcriptional, chemosensory and cell-contact-dependent regulation of type IV pilus expression. *Current Opinion in Microbiology* 5:173-178.
- Wolfgang, M., Park, H.S., Hayes, S.F., van Putten, J.P.M., and Koomey, M. 1998. Suppression of an absolute defect in type IV pilus biogenesis by loss-of-function mutations in *pilT*, a twitching motility gene in *Neisseria gonorrhoeae*. *Proceedings of the National Academy of Sciences of the United States of America* 95:14973-14978.
- Zaini, P.A., Fogaca, A.C., Lupo, F.G.N., Nakaya, H.I., Vencio, R.Z.N., and da Silva, A.M. 2008. The iron stimulon of *Xylella fastidiosa* includes genes for type IV pilus and colicin V-like bacteriocins. *Journal of Bacteriology* 190:2368-2378.

Chapter 4 Calcium promotes *X. fastidiosa* colonization and virulence in a model plant host

Abstract

Colonization of plant xylem vessels and formation of bacterial biofilms that result in the obstruction of water and mineral nutrients is thought to be part of the virulence mechanisms of the plant pathogen *Xylella fastidiosa*. Alterations in the plant mineral content of infected plants compared to uninfected plants have been previously reported. Among them, Ca accumulation is consistently identified in plants hosts both experimentally and naturally infected with *X. fastidiosa*. In addition, the impact of Ca on bacterial virulence determinants including attachment, aggregation, biofilm production, and twitching motility in vitro has been demonstrated by this research group. Here, using the model system *Nicotina tabaccum* SR1, the effects of Ca fertilization on the development of host symptoms, bacterial populations, and host tissue Ca accumulation was examined. Our results indicate that Ca fertilization increases bacterial populations and promotes symptom development. Moreover, *X. fastidiosa*-induced Ca accumulation was observed for Ca-supplemented and non-supplemented treatments and was more pronounced than Ca accumulation due to fertilization alone. These results suggest that Ca is a key factor in *X. fastidiosa*-host plant interactions, and it might be involved in the mechanism of *X. fastidiosa* pathogenicity. Thus, *X. fastidiosa* infection induces an increase in the Ca uptake in the plant host, which in turn promotes further colonization by the bacterium.

Introduction

Xylella fastidiosa is an insect-vectored, plant-pathogenic bacterium that exclusively inhabits the xylem vessels of the plant host (Davis et al., 1978; Almeida and Purcell, 2003). *X. fastidiosa* is able to infect several hosts causing symptomatic or asymptomatic infections (Hopkins and Purcell, 2002). Symptoms among the most economically-important hosts may include interveinal chlorosis, leaf marginal necrosis, and small fruits in citrus; dwarfed leaves and fruits and their premature fall in coffee; stunted plant with shortened stem internodes in peach and alfalfa; leaf marginal necrosis, leaf blade drop with stem still attached to the stem, and fruit mummification in grape; leaf scorch in oleander, blueberry, almond, pecan elm, oak, sycamore, and maple (Hopkins and Purcell, 2002; Stevenson et al., 2005).

The exact mechanism of *X. fastidiosa* pathogenicity is not known. Symptoms are thought to result from water deficit as a consequence of vessel obstruction caused by bacterial colonization. Bacterial production of biofilms (cells embedded in an exopolysaccharide matrix) and plant host formation of tyloses (outgrowths on parenchyma cells of xylem) and gums, as host-defense responses to invasion, cause this vessel obstruction (Purcell and Hopkins, 1996; Hopkins and Purcell, 2002; Stevenson et al., 2004). However, this hypothesis is controversial. The role of water deficit in disease development among different plant species has not been consistent (McElrone et al., 2003; Thorne et al., 2006), and no correlation has been shown between the quantity of bacteria and number of colonized and blocked vessels and symptom severity (Newman, 2003; Gambetta et al., 2007). Although the role of water deficit in development of diseases caused by vascular pathogens has been documented (Yadeta and BP, 2013), low water status does not completely explain the physiological changes suffered by plants infected with *X.*

fastidiosa. Comparisons between plants experimentally exposed to water deficit and plants infected with *X. fastidiosa* show quantitative and qualitative differences in symptoms (Thorne et al., 2006), indicating water deficit may not be the sole cause of observed symptoms.

Collectively, some studies suggest potential alternative explanations for disease development involving the role of mineral element acquisition by *X. fastidiosa*. A previous study identified the importance of the xylem sap chemistry on the ability of the bacteria to attach to surfaces and form aggregates (Zaini et al., 2009), both of which are important features for biofilm formation, a key determinant of bacterial virulence (Caserta et al., 2010). A positive correlation between zinc and copper levels and the amount of planktonic growth and a correlation between calcium levels and the amount of biofilm formation were found when sap from susceptible and resistant grape cultivars were compared (Andersen et al., 2007). Analysis of xylem vessels occluded with *X. fastidiosa* showed accumulation of Ca and Mg, which are suggested to mediate cell attachment by acting as cation bridges between the negatively charged bacteria and negatively charged xylem (Leite et al., 2002). The role of Ca in the regulation of attachment, aggregation, biofilm formation, and twitching motility of *X. fastidiosa* has been indicated (Cruz et al., 2012 Chapter 1). In addition, Fe has been shown to control the expression of genes implicated in bacterial virulence, such as type IV pili genes and genes encoding the bacteriocin, colicin (Zaini et al., 2008). Moreover, biofilm cells accumulate higher levels of Ca along with other elements including Cu, K, Mn, and Zn, compared to planktonic cells (Cobine et al., 2013).

Furthermore, infection with *X. fastidiosa* has been shown to impact the nutritional status of the plant host. Analyses of citrus plants with symptoms of *X. fastidiosa* infection indicated

deficiencies in P, K, and Zn and high concentrations of Fe and Mn compared to non-infected plants (Silva-Stenico et al., 2009). In addition, ionome analyses (measurement of the total elemental composition of an organism) of leaves of tobacco plants, used as a model host system, indicated significant increases in the accumulation of Ca in infected versus non-infected leaves and a reduction in the accumulation of P (De La Fuente et al., 2013). The same study compared the ionome of *X. fastidiosa*-infected and non-infected leaves collected in the field from different cultivars of grape, blueberry, and pecan. The results of ionome analyses showed the same tendencies: higher accumulation of Ca and reduction in P in symptomatic leaves (De La Fuente et al., 2013).

The aim of this study was to determine the effect of Ca supplementation on the pathogenicity of *X. fastidiosa* in the model plant host *Nicotiana tabacum*. Plants inoculated with *X. fastidiosa* and fertilized with different concentrations of Ca were monitored over time to determine the impact of Ca on bacterial populations and symptom development. Results indicate a correlation between Ca accumulation in plant leaves and bacterial concentrations and symptom severity, suggesting a role of this element in bacterial pathogenicity.

Materials and Methods

Plant material and greenhouse conditions

The experiment was carried out in the greenhouse at 20–25°C with natural sunlight. *Nicotiana tabacum* ‘Petite Havana SR1’ seeds (Plant Introduction (PI) number 552516) were obtained from the USDA Germplasm Resources Information Network (GRIN) and were germinated in

Sunshine Mix #8 (Sun Gro Horticulture Canada Ltd., Vancouver, Canada). One month-old seedlings were transplanted and grown in individual 4.5-inch square pots. Three weeks after transplanting, 5-leaf stage plants were inoculated with *X. fastidiosa* as previously described (Francis et al., 2008). Tobacco plants were prepared for inoculation by cutting the top of the stem, leaving three remaining leaves at the lower portion of the plant. For the inoculation, *X. fastidiosa* WM1-1 (Parker et al., 2012), were grown on Periwinkle Wilt (PW) (Davis et al., 1981) media at 28 °C for one week, scraped off, suspended in succinate-citrate-phosphate buffer, and adjusted to a concentration of 1×10^8 CFU/mL. The bacterial suspension or sterile buffer was inoculated with a 1 ml tuberculin syringe in the axils of the three basal leaves of each plant.

One day after inoculation, plants were subjected to three different Ca supplementation treatments: 150 ml of water (0mM Ca), 3 mM CaCl_2 solution, or 8 mM CaCl_2 solution was applied to the substrate of each plant once per week for the duration of the experiment (11 weeks). Additional watering was done with non-amended tap water. A total of twenty plants, ten inoculated and ten buffer-inoculated control plants, were used per Ca supplementation treatment, the experiment was conducted one time.

When symptoms started to appear ~ 8 weeks after inoculation, all leaves were collected from 10 plants per watering treatment; five inoculated and five buffer-inoculated control-plants. The remaining plants were sampled in a second time point 11 weeks after inoculation. Plants were fertilized with Peters Professional 20-10-20 Peat-Lite Special (The Scotts Company, Marysville, OH), three times during the experiment.

Bacterial population quantification

A portion of each leaf petiole (approximately 250 mg) was excised and weighed. DNA was extracted from each sample using a modified cetyltrimethylammonium bromide (CTAB) protocol (Doyle and Doyle, 1987) and suspended in 50 µl of molecular-grade water. From these DNA samples, bacterial populations were determined using quantitative PCR (qPCR). qPCR was performed using the previously designed HL5/HL6/HLP set of *X. fastidiosa*-specific primers and TaqMan probe (Francis et al., 2006). qPCRs (20µl total volume) consisted of 1X ABsolute Blue QPCR ROX mix (ABgene-Thermo Fisher Scientific, Surrey, United Kingdom), 0.4 µM probe (5'- 6FAM, 3'-BHQ1), 0.2 µM each primer, and 1 µl DNA. Samples were amplified on an ABI 7500 Real-Time PCR system (Applied Biosystems, Foster City, CA), using the following cycling parameters: 95°C for 1 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. All qPCR runs included a 4-point standard curve prepared from 10-fold serial dilutions of DNA extracted from an *X. fastidiosa* cell suspension of a known concentration. Cells in the suspension were enumerated by spread plating serial dilutions onto PW agar and counting the CFU produced after two weeks of incubation at 28°C. qPCR amplification efficiencies were comparable to those described previously (Francis et al., 2006). CFUs obtained by qPCR were then divided by the petiole tissue sample weight, to obtain CFU/mg of tissue.

Plant mineral content analysis

The mineral content of plant material was determined as described previously (De La Fuente et al., 2013). Leaves were dried at 65 °C for 1 hour, crushed to a fine powder and two samples of

approximately 5 and 10 mg were used for the analysis (exact weight was recorded). Samples were digested for 1 hour at 100 °C in 200 mL of mineral-free concentrated nitric acid (OPTIMA, Fisher Scientific), centrifuged at 13,000xg for 2 min, and stored at room temperature overnight. Digested samples were diluted with 800 µl of ultra-pure, mineral-free water and centrifuged at 13,000xg for 2 min to remove any remaining particulates. Samples were analyzed by inductively coupled plasma with optical emission spectroscopy (ICP-OES, Perkin Elmer 7100 DV, Waltham, MA) with simultaneous measurement of Ca, Co, Cu, Fe, K, Mg, Mn, Mo, Na, P, S, and Zn. Mineral concentrations were determined by comparing emission intensities to a standard curve created from certified mineral standards (SPEX CertiPrep, Metuchen, NJ).

Statistical analysis

Bacterial population

Analysis of variance (ANOVA) was performed using SAS version 9.3 (SAS institute INC., Cary, North Carolina, USA). The response variable “Number of CFUs in the leaf” was analyzed using Proc Glm using a completely randomized design (CRD). The numbers of CFUs per leaf were logarithmically transformed in order to meet the assumptions of the ANOVA (normal distribution and equal variances). Main factor effects, time point, calcium level, and leaf position, and their possible interactions were also considered in the analysis. In addition, linear contrast analyses were made in order to identify difference between the calcium levels. Finally, the overall ANOVA was split by each of the main effects and levels in order to elucidate the impact of those in the response variable.

Mineral content

Analysis of variance (ANOVA) was performed using SAS version 9.3, (SAS institute INC., Cary, North Carolina, USA). The response variable “Calcium content” was analyzed using Proc Glm using a completely randomized design (CRD). Residuals from the collected data were analyzed in order to confirm normal distribution and meet the assumptions of the ANOVA. Main factor effects, time point, inoculation, and calcium level, and their possible interactions were also considered in the analysis. In addition, the overall ANOVA was split by each of the main effects and levels in order to elucidate the impact of these factors on the response variable calcium content.

Results

Symptoms development

Initial symptoms, 8 weeks after inoculation, included small chlorotic lesions located around the main and secondary veins for all the Ca levels. No symptoms were observed on the non-inoculated control plants. At this time point, the treatment with the higher number of leaves exhibiting disease symptoms (chlorotic spots) was 8 mM CaCl_2 (80% of leaves affected), followed by the non-Ca control treatment (40%) and 3 mM CaCl_2 (24%). For the second time point, 100% of the leaves in all the treatments present extensive interveinal chlorosis (Figure 4-1A); however, only Ca-supplemented plants (3 mM and 8 mM) showed leaf wilting and leaf curling, the latter of which was observed exclusively in the top leaves (Figure 4-1A). For both 3 mM and 8 mM Ca treatments, 75% of the leaves were wilted. None of these symptoms were observed in the non-inoculated control plants (Figure 4-1B).

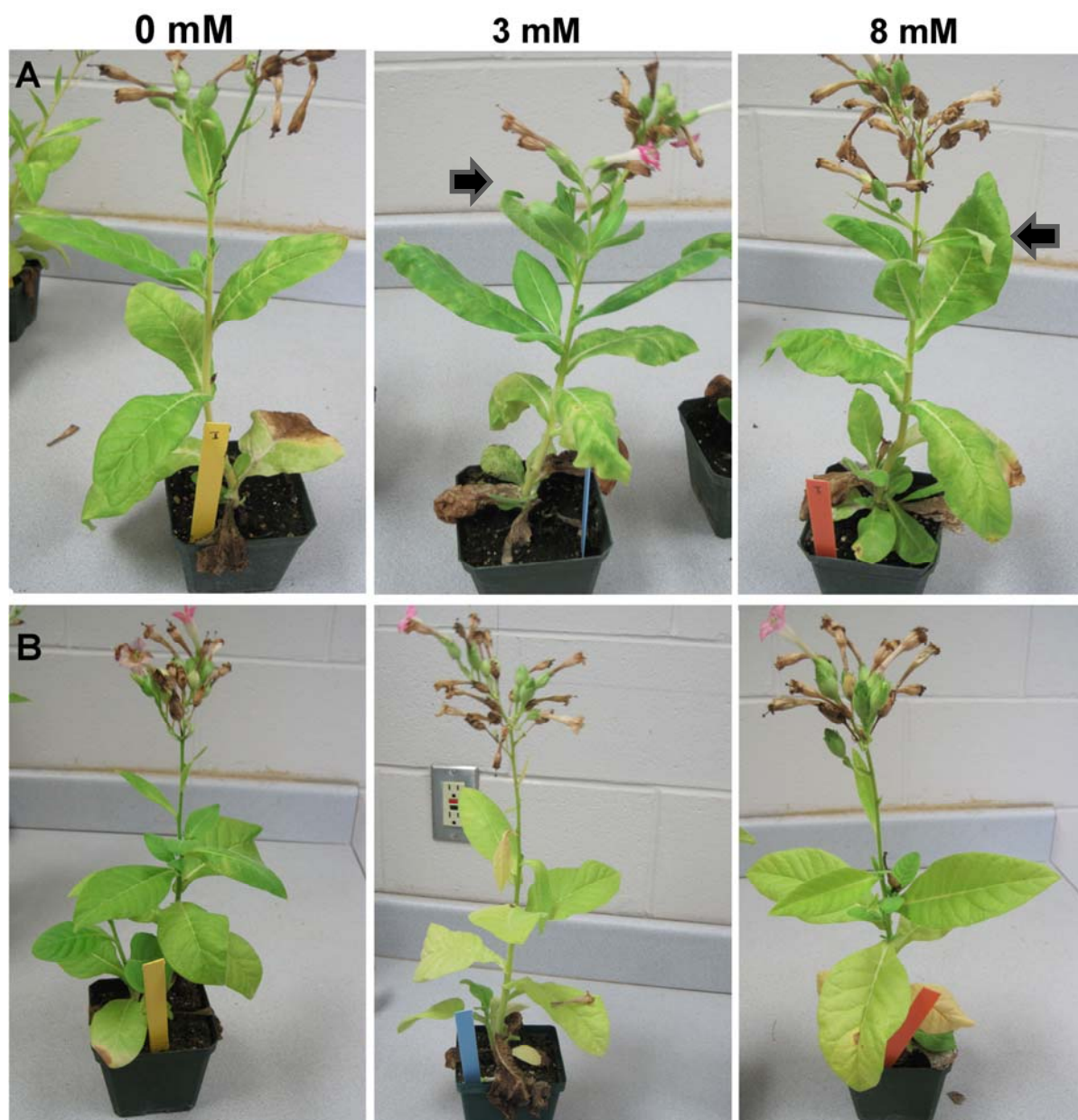


Figure 4-1.Effect of Ca fertilization on *X. fastidiosa* disease symptoms.

Columns correspond to the Ca supplementation treatment (0, 3 or 8 mM CaCl_2 added to plants once a week. A) Plants showing representative symptoms at the second time, leave curling is indicated by a black arrow. B) Control non-inoculated plants.

Changes in Ca accumulation in *X. fastidiosa* infected and non-infected tobacco

Overall analysis of leaf Ca concentration considering both time points, including inoculated and non-inoculated leaves, indicated that inoculation ($P<0.0001$), Ca level ($P<0.0001$), and the interaction of both factors ($P<0.0001$) were significant sources of variation in the experiment.

Accumulation of Ca due to its supplementation was determined by comparing Ca content among the three levels of Ca in non-inoculated plants. In the first time point, significantly higher accumulation was found for 8 mM Ca ($P= 0.0124$) compared with the plants in the non-supplemented treatment. Non-significant differences were 3 mM Ca and 0 mM ($P= 0.051$) and, non-significant differences between the 3 mM and 8 mM treatments ($P= 0.578$) were observed. In the second time point, 3 mM($P= 0.002$) and 8 mM($P= 0.0001$)-treated plants showed higher accumulation of Ca than the non-supplemented control and, non-significant differences between the 3 mM and 8 mM treatment ($P= 0.1663$) were found (Fig 4-2). No phenotypically differences were detected for the plants under Ca supplementation for the first sampling point and in the second time point; non-inoculated plants showed yellowing faster than the inoculated plants.

Further analysis to determine the changes in Ca accumulation due to the bacterial infection were done by comparing inoculated versus non-inoculated plants. At time point-one, significantly highest Ca concentrations were detected in the non-Ca-treated plants, with 50.2% increase ($P<0.0001$) followed by 8 mM Ca with 27.6% increase ($P<0.0001$). Non-significant differences between inoculated and non-inoculated plants were indicated for the 3 mM Ca treatment, which resulted in an increase of 13.1% ($P=0.195$) (Fig 4-2). The same analysis for the second time

point indicated significantly higher Ca accumulation in the inoculated compared to non-inoculated plants for all the treatments. Plant from the non-Ca- supplemented treatment showed 139.8% ($P<0.0001$), 3 mM Ca 68.7% ($P<0.0001$) and 8 mM 89.4% ($P<0.0001$) (Figure 4-2).

To establish a correlation between the effect of Ca fertilization and accumulation of this mineral due to the bacterial infection, the content of Ca was compared in inoculated plants among the three Ca levels. In the first time point, non-significant differences among the three Ca levels were found (Figure 3). For the second time point, the 8 mM Ca treatment showed significantly higher accumulation of Ca compared with the 3 mM treatment ($P= 0.001$) and the non-Ca treatment ($P= 0.0005$). In contrast, no differences were found between 3 mM and non-Ca-supplemented treatment ($P= 0.655$) (Figure 4-2).

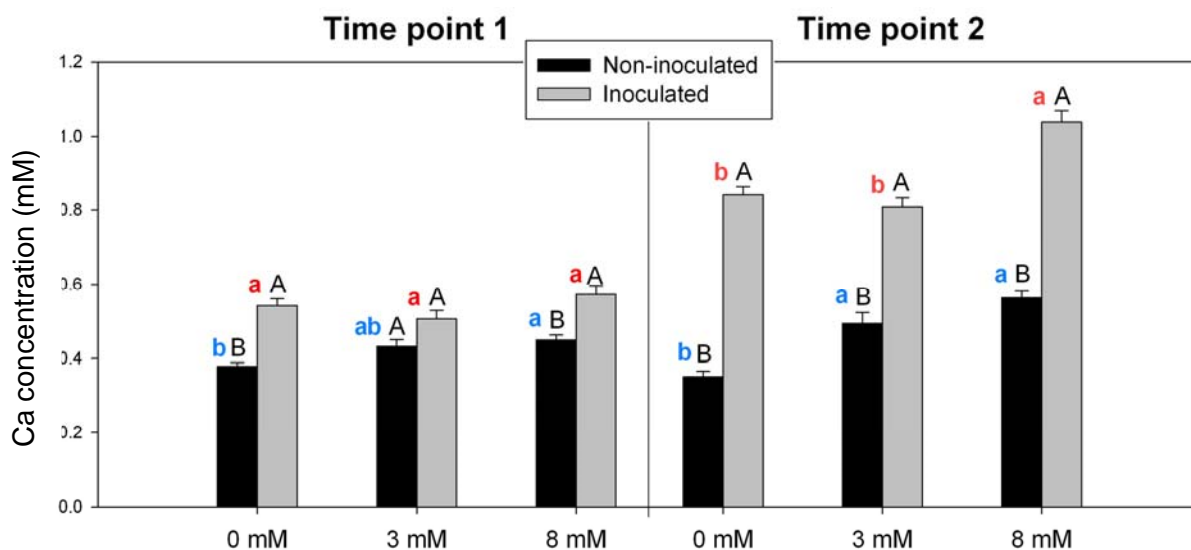


Figure 4-2. Concentration of Ca in leaves of *X. fastidiosa*-inoculated and non-inoculated plants under three levels of Ca fertilization.

Five leaves per plant of five plants per treatment were collected in for each time point and mineral content was analyzed by ICP-OES. Different letters on the bars indicate significant

differences ($P \leq 0.05$), capital letters indicate statistical differences comparing inoculated vs. non-inoculated plants in the same time point, lower case letter indicate statistical differences of either inoculated or non-inoculated among the different Ca levels in the same time point. Error bars represent the standard error of the mean (n=25).

Effect of *X. fastidiosa* in the accumulation of other mineral elements

To determine the effect of *X. fastidiosa* infection on the mineral content of the plants, tissues from inoculated versus non-inoculated plants were compared. In time point one, significant increases in accumulation were observed for: B for the 3 Ca levels No Ca (42.2%) ($P= 0.0001$), 3 mM (17.95%) ($P= 0.004$) and 8 mM (15.9%) ($P= 0.004$); Cu for No Ca (100.5%) ($P= 0.001$) and 8 mM ($P= 0.0002$); Mg for the three levels No Ca (47.2%) ($P< 0.0001$) 3 mM (17.3%) ($P=0.0095$) and 8 mM (15.6%) ($P< 0.006$); Mn for No Ca (131%) ($P< 0.0001$) and, S for No Ca (60.94%) (< 0.0001). In contrast, significant decreases in the accumulation was determined for K in the 3 Ca levels No Ca (13.1%) ($P= 0.036$) 3 mM (18.4%) ($P= 0.0085$) 8 mM (% 22.7) ($P< 0.0001$). In the second time point significant differences were observed in B for the 3 Ca levels No Ca (113.6%) ($P= 0.0001$), 3 mM (63.05%) ($P= 0.0001$) and 8 mM (87.02%) ($P= 0.0001$); Mg for the levels no Ca (113.6%) ($P<0.0001$) and 3 mM (53.7%) ($P= 0.0001$) and 8 mM (53.7%) ($P= 0.0001$); Mn for the 3 Ca levels Ca (90.3%) ($P= 0.0001$), 3 mM (138.9%) ($P= 0.0001$) and 8 mM (15.6%) ($P= 0.008$); Na for No Ca (67.9%) ($P= 0.0007$) and 3 mM (58.0%) ($P= 0.004$); P for no Ca (72.7%) ($P< 0.0001$) and 8 mM (64.69%) ($P= 0.01$) and S in the three levels No Ca (59.8%) ($P= < 0.0001$), 3 mM (93.1%) and 8 mM ($P= < 0.0001$) (101.3%) ($P= 0.0009$) (Figure 4-3).

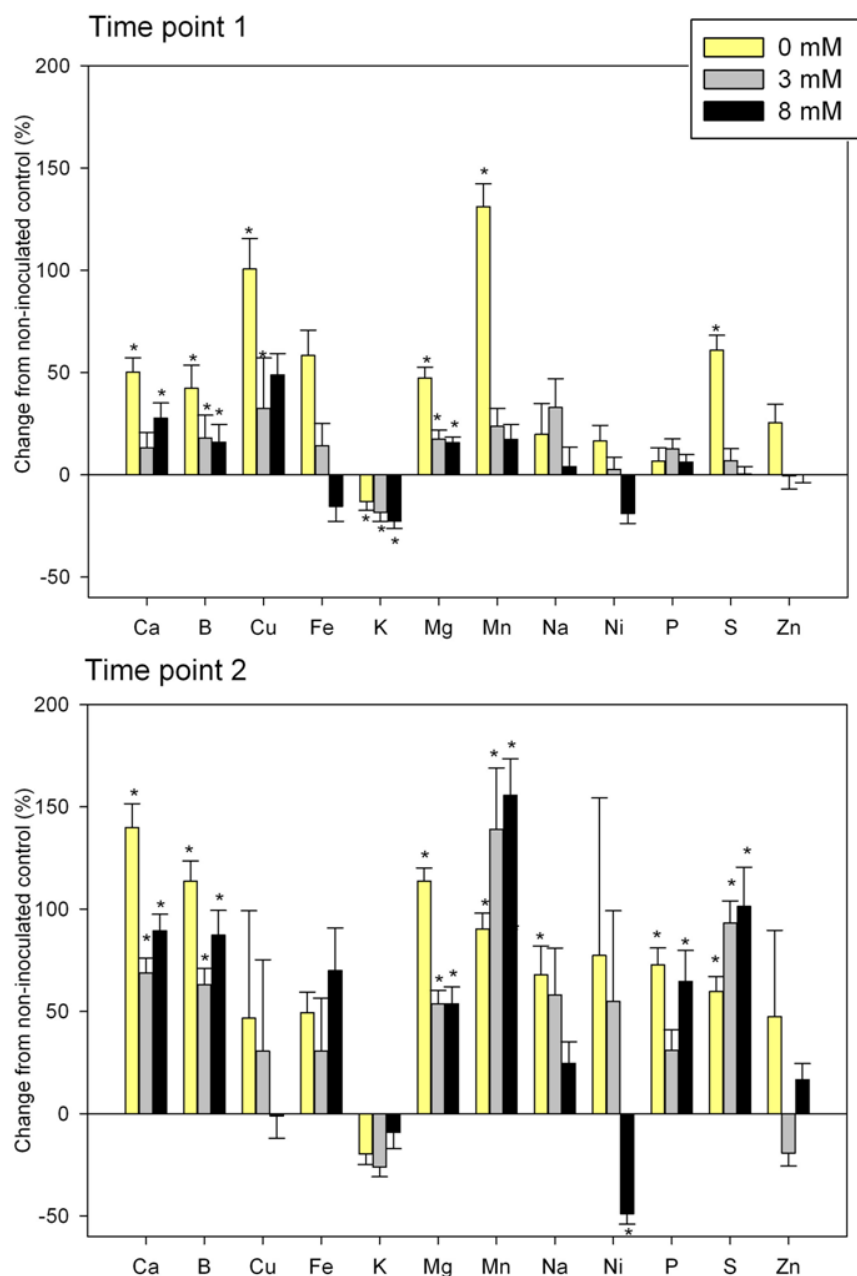


Figure 4-3. Changes in the mineral composition of tobacco leaves infected with *X. fastidiosa* under 3 levels of Ca fertilization.

Percentage of change was calculated by comparing the mean values of inoculated against non-inoculated, independently for each time point. Five leaves per plant of five plants per treatment were collected in for each time point and mineral content was analyzed by ICP-OES. Asterisk

the bars indicate significant differences ($P \leq 0.05$). Error bars represent the standard error of the mean (n=25).

Effect of Ca fertilization in the plant ionome

To determine any changes in the accumulation of other metals due to Ca fertilization, non-inoculated plants supplemented with 3 mM Ca and 8 mM Ca were compared to the non-supplemented control. For the time point one, significant increase in the concentration of: Cu in 3 mM Ca by 40.8% ($P=0.01$) and 8 mM by 34.3% ($P=0.04$), Fe in 8 mM by 29.5% ($P=0.001$), Mg in 8 mM by 18.8% ($P=0.006$), Mn in 8 mM ($P=0.01$) by 24.1%. While a significant reduction in the concentration of K in 3 mM by -12.5% ($P=0.03$) and P in 3 mM by 11.5% ($P=0.002$). In the second time point significant increase was found for Mg in 8 mM by 31.6% ($P=0.02$) and Ni in 3 mM and 8 mM by 99.1% ($P=0.002$) and 85.8% ($P=0.01$). In contrast, a significant reduction in S of 38.5% was found in 8 mM treatment (Figure 4-4).

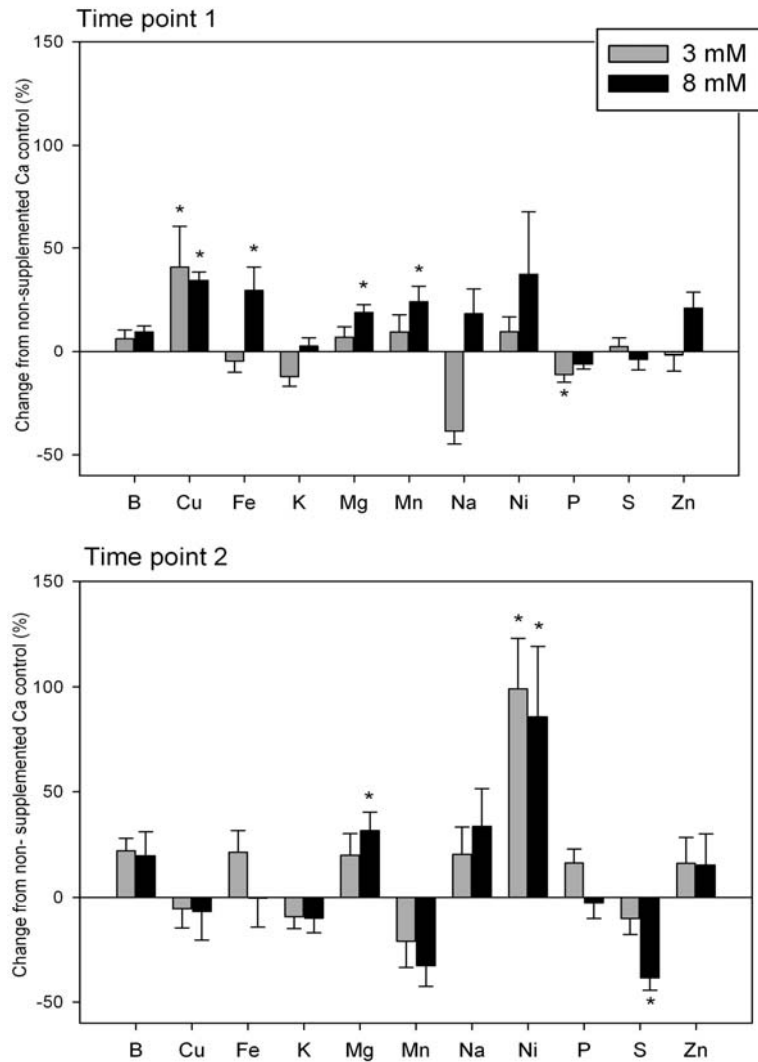


Figure 4-4. Changes in mineral composition of tobacco plants, under Ca fertilization.

Percentage of change was calculated by comparing the mean values of either 3 mM or 8 mM Ca supplemented treatments to the non-supplemented treatment in the non-inoculated leaves. Five leaves per plant of five plants per treatment were collected in for each time point and mineral content was analyzed by ICP-OES. Asterisks on the bars indicate significant differences ($P \leq 0.05$), capital letters indicate statistical differences. Error bars represent the standard error of the mean ($n=25$).

Effect of Ca on the bacterial populations of tobacco infected plants

General analysis of the data obtained from quantification of bacterial populations in the first time point indicated that Ca level had a highly significant effect on the bacterial population ($P<0.0001$), while leaf position ($P=0.718$) and calcium level*leaf position interaction ($P=0.693$) were non-significant sources of variation at this time point. Comparison of bacterial populations present in the different Ca treatments indicated that the populations present in 3 mM and 8 mM Ca treatments were significantly larger than those present in non-Ca supplemented control plants ($P<0.0001$). Non-significant differences were observed between the 3 mM and 8 mM Ca treatments ($P=0.648$) (Figure 4-5 A). Comparisons of the bacterial populations among the different five leaf positions were non-significantly different in any of the Ca levels (Figure 4-5B).

Similarly, for the second time point, analysis of bacterial populations indicated that the overall Ca level had a significant effect ($P<0.0001$) on the bacterial populations, while leaf position ($P=0.531$) and calcium level*leaf position interaction ($P=0.201$) were non-significant sources of variation for this time point. In contrast to the first time point, bacterial populations present in all the Ca level were significantly different ($P<0.0001$) (Figure 4-5C). Bacterial populations among the five leaf positions showed non-significant differences (Figure 4-5 D). The bacterial population present in the non-Ca supplemented plants showed a significant increase from the first time point to the second time point time point two ($P<0.0001$), populations in the 3 mM Ca treatment did not change ($P=0.652$), while a significant decrease was exhibited by the population in 8 mM Ca treatment ($P=0.0005$)(Figure 4-5 A,C).

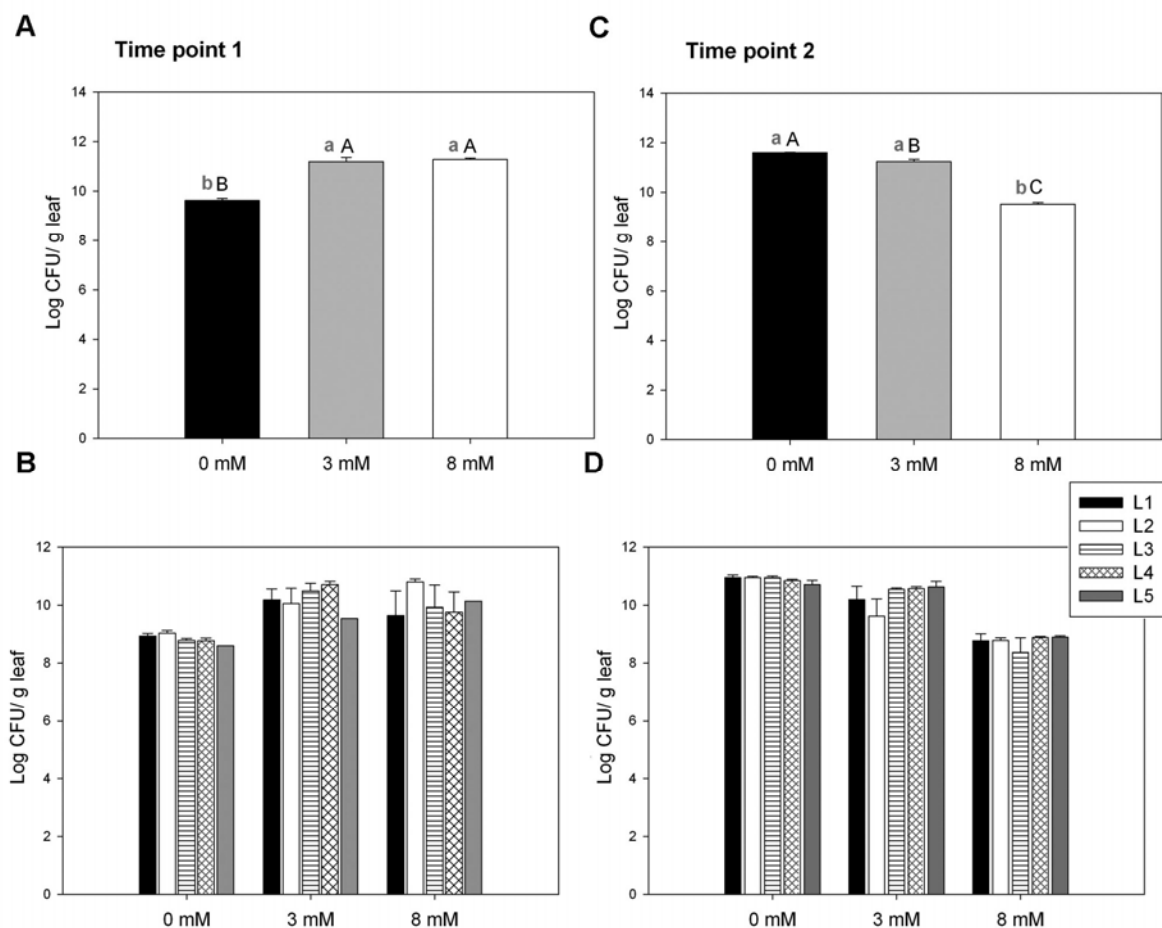


Figure 4-5. Bacterial population of tobacco plants infected with *Xylella fastidiosa*.

A, C) Bacterial populations in Ca supplementation treatments were obtained by adding the populations in five leaves per plant, the data represented in the graph are the average of five plants per treatment in the two time points B, D) Bacterial populations per leaf position, corresponds the average of the bacterial population of five leaves from five plants in each treatment. Different letters on the bars indicate significant differences ($P \leq 0.05$), capital letters indicate statistical differences among the different treatments at the same time point, lower case letter indicate statistical differences in the same Ca level for in the two time points. Error bars represent the standard error of the mean (n=5).

Discussion

The precise mechanism by which *X. fastidiosa* causes disease is still under investigation. *X. fastidiosa* encodes cell-associated proteins and exopolysaccharides implicated in attachment and twitching motility, which are directly linked to the formation of biofilms (da Silva et al., 2001; Meng et al., 2005; De la Fuente et al., 2007; Li et al., 2007) thought to be responsible for the reduction in water conductance that favors the disease symptoms (Stevenson et al., 2004).

It is known that water stress is a component of the disease, but water stress does not explain all the physiological changes of the host during infection. Indeed, plant responses to water stress differ from those of infection observed in expression of symptoms as well as the in patterns of gene expression (Choi et al., 2013). Another characteristic observed during infection is the changes in the mineral status of the plant, which could be either a direct or indirect result of bacterial infection (Andersen and French, 1987; Goodwin et al., 1988; Silva-Stenico et al., 2009; De La Fuente et al., 2013). Among other metals, different reports have coincided in a relationship between the infection of *X. fastidiosa* and the accumulation of Ca (Goodwin et al., 1988; Leite et al., 2002; De La Fuente et al., 2013). A previous study demonstrated this tendency in naturally infected plants of grapevine, blueberry and pecan and, experimentally infected tobacco, as well as in experimentally-infected tobacco plants (De La Fuente et al., 2013). Here, the effect of different levels of Ca fertilization on symptom development and bacterial populations in tobacco plants was examined. Results suggest that Ca has a role in *X. fastidiosa*-host interactions. Ca fertilization increases the bacterial population and the development of symptoms, and, in turn, bacterial infection promotes Ca uptake.

Analysis of the content of Ca in inoculated versus non-inoculated plants, in all the Ca levels tested here, indicated greater accumulation of Ca in the inoculated plants; this is in agreement with a previous report (De La Fuente et al., 2013). Besides the effect caused by bacterial infection and the fertilization treatment, additional increase in Ca accumulation due to the fertilization-bacterial infection interaction was only observed in the 8 mM Ca treatment at the second time point. This indicates that the uptake of Ca, even when the bacterium is present, is limited by the plant accumulation capacity at a given developmental stage and/ or by stomatal transpiration. Stomatal transpiration depends on various factors including light and temperature (Marschner, 1995), which could vary between the two sampling points and is reflected in a lower percentage of increase at the first time point.

The plant or bacterial mechanisms causing the increase of Ca in *X. fastidiosa*-infected plants require further investigation. Reports in animal and plant-pathogenic bacteria have demonstrated the bacterial ability to manipulate host Ca levels as a strategy for bacterial invasion. For instance, the type IV pili subunit of *Neisseria* spp. trigger an elevation in the host cytosolic Ca upon cell contact, which is required for posterior bacterial cell attachment (Kallstrom et al., 1998). Apoplastic Ca chelation by bacterial EPS is another virulence mechanism used to avoid the host defense-response (Aslam et al., 2008). The well-studied function of Ca as a secondary messenger requires the influx of extracellular Ca as a prerequisite for the induction of innate immunity, the basic level of defense response (TranVan Nhieu et al., 2004).

The Ca accumulation observed at the whole plant level in these experiments differs from the characteristic transient cytosolic oscillations in Ca concentration involved in signaling pathogen

invasion or abiotic stress (Lecourieux et al., 2006). Instead, the increase in the accumulation observed here seems to be long-term plant uptake that results in the observed changes. The location of Ca accumulation could not be predicted by this experiment. However, it should be extracellular given the cytotoxic nature of this element (Campbell, 1990). In leaves, Ca is particularly abundant in the cell walls due to the formation of calcium pectates in the middle lamella (Marschner, 1995). Under high supply conditions, prevention of excessive accumulation of Ca is achieved by the formation of insoluble oxalate crystals (Marschner, 1995). The presence of these crystals has been previously reported in grape (Fritschi et al., 2008), coffee (Queiroz-Voltan et al., 1998), and citrus (Alves et al., 2009) infected with *X. fastidiosa*.

Quantification of bacterial populations indicated that host supplementation with Ca favors bacterial colonization. Larger bacterial populations in Ca-supplemented plants than in non-supplemented plants were identified in samples collected at the first time point. In contrast, quantification of bacterial population at the second time point indicated a decline in the population in the Ca-supplemented treatment, as the concentration in Ca treatment increased. This can be explained by an initial rapid increase in the bacterial number in Ca-supplemented treatments that overload the plant capacity, followed by a population decay observed at the second time point. While slower growth in the non-supplemented treatment, which was observed as smaller numbers in the first time point, allowed the population reach the number observed in the second time point. At the basic level, Ca functions in prokaryotic cells includes roles in cell cycle and cell division and integrity of the cell wall and lipopolysaccharide (Norris et al., 1996; Yu and Margolin, 1997; Onoda et al., 2000; Dominguez, 2004). In addition, in *X. fastidiosa* and other bacterial species, Ca can benefit bacterial colonization by improving attachment (Cruz et

al., 2012 Chapter 1), promoting production of EPS (Patrauchan et al., 2005; Patrauchan et al., 2007), and promoting biofilm formation and stabilization (Fletcher, 1988; Rose, 2000; Leite et al., 2002). These facts may explain the observed increase in population numbers. In this experiment, a positive correlation between the appearance of wilting symptoms and the bacterial populations was observed. However, the appearance of chlorotic spots was not correlated with this parameter; by the end of the experiment the severity of chlorosis in leaves was comparable in all the treatments. Bacterial populations and their correlation with water deficit and symptoms development have been controversial, as high numbers of bacteria or bacteria-colonized vessels has not always coincided with severe leaf symptoms (Newman, 2003; Thorne et al., 2006; Gambetta et al., 2007). The results of this work intuitively allow the associations of high bacterial populations with vessel blockage resulting in water deficit and wilting. However, this is not considering the populations of individual leaves and its association with specific symptoms in specific location. Even with uniform distribution of bacterial populations among the different leaf positions, as the observed in this experiment, this correlation (bacterial population- water deficit) should be carefully considered.

Analysis of other metals to establish changes in inoculated compared with non-inoculated plants indicated that significant changes in the accumulation of different metals detected in any of the Ca treatments were always also detected in the non-supplemented control. This suggests that these changes are due to bacterial infection rather than to Ca fertilization treatment. Except by Fe and Zn, all the micro and macro nutrients analyzed showed to change in at least one of the time points. De la Fuente et al. reported only an increase in Ca and a decrease in P in similar experiments using tobacco plants (De La Fuente et al., 2013). However, those experiments were

conducted using *X. fastidiosa* strain Temecula, a different strain than that used in the present study. In addition, the mineral content analyses conducted in that work correspond to a pool of five time points.

Results of this experiment showing a general change in all the metals suggested that the disease is causing a metabolic disorder that involves a reduction in the selectivity of the roots to ions, in addition to some changes consistent with water stress. Other studies comparing *X. fastidiosa*-infected and healthy plants detected increases in Ca and Mg and reductions in K in grapevines (Goodwin et al., 1988); increases in Fe Mn and Zn and decreases in P and K in citrus (Silva-Stenico et al., 2009); and increases in Mn and Na and decreases in P, K, B, and Mo in peach (Andersen and French, 1987). The level of P observed in this experiment differs from previous reports. In contrast, in accordance with previous studies, a decrease in K was detected in the present work. K plays a main role in the cellular water status (Marschner, 1995), and this decrease correlates with the observed hydric stress of infected plants. Other alterations in the mineral composition including accumulation of Mg and Na were also reported in water stressed plants (McElrone et al., 2003).

Though the changes in the plant mineral accumulation seemed to be due to the bacterial infection, further analyses were conducted in order to determine any indirect effects of Ca fertilization. Most of these changes include increases in the micronutrients Cu, Fe, and Mn. These effects have been previously observed in tobacco under Ca fertilization (López-Lefebvre et al., 2001), changes in these metals were only observed in the first time point. Other metals found to be altered by Ca fertilization include Mg, P, and Ni. It is known that Ca reacts with P to form

insoluble compounds, and this reaction affects the availability of P more than it does the availability of Ca (Datnoff et al., 2007). Ca also antagonizes the uptake of Mg by competing for the binding sites at the plasma membrane and exchange sites at the cell walls (Marschner, 1995); however, an increase of Mg was observed at two time point.

Several previous studies have documented the role of Ca enhancing plant disease resistance, explained for its structural role as a component of the primary cell wall, crucial for plant strength and the inhibition of microbial enzymes (Datnoff et al., 2007), and its role as a secondary messenger (Hepler, 2005). In contrast, here, it is hypothesized that *X. fastidiosa* has developed a mechanism in which it manipulates host physiology in order to increase Ca uptake, promoting bacterial infection rather than benefiting the host. In general, alteration in the host mineral status seems to be a virulence strategy of this bacterium. This information can be used in the development of disease management strategies, such as in plant nutritional programs designed to restrict the development of bacterial populations and the development of symptoms.

References

- Almeida, R.P., and Purcell, A.H. 2003. Transmission of *Xylella fastidiosa* to grapevines by *Homalodisca coagulata* (Hemiptera: Cicadellidae). *Journal of economic entomology* 96:264-271.
- Alves, E., Leite, B., Pascholati, S.F., Ishida, M.L., and Andersen, P.C. 2009. Citrus sinensis leaf petiole and blade colonization by *Xylella fastidiosa*: details of xylem vessel occlusion. *Sci Agr* 66:218-224.
- Andersen, P.C., and French, W.J. 1987. Biophysical characteristics of peach trees infected with phony peach disease. *Physiological and Molecular Plant Pathology* 31:25-40.
- Andersen, P.C., Brodbeck, B.V., Oden, S., Shriner, A., and Leite, B. 2007. Influence of xylem fluid chemistry on planktonic growth, biofilm formation and aggregation of *Xylella fastidiosa*. *Fems Microbiology Letters* 274:210-217.
- Aslam, S.N., Newman, M.A., Erbs, G., Morrissey, K.L., Chinchilla, D., Boller, T., Jensen, T.T., De Castro, C., Ierano, T., Molinaro, A., Jackson, R.W., Knight, M.R., and Cooper, R.M. 2008. Bacterial polysaccharides suppress induced innate immunity by calcium chelation. *Current biology* : CB 18:1078-1083.
- Campbell, A.K. 1990. Calcium as an intracellular regulator. *Proceedings of the Nutrition Society* 49:51-56.
- Caserta, R., Takita, M.A., Targon, M.L., Rosselli-Murai, L.K., de Souza, A.P., Peroni, L., Stach-Machado, D.R., Andrade, A., Labate, C.A., Kitajima, E.W., Machado, M.A., and de Souza, A.A. 2010. Expression of *Xylella fastidiosa* fimbrial and afimbrial proteins during biofilm formation. *Appl Environ Microbiol* 76:4250-4259.
- Choi, H.K., Iandolino, A., da Silva, F.G., and Cook, D.R. 2013. Water deficit modulates the response of *Vitis vinifera* to the Pierce's disease pathogen *Xylella fastidiosa*. *Molecular plant-microbe interactions* : MPMI 26:643-657.
- Cobine, P.A., Cruz, L.F., Navarrete, F., Duncan, D., Tygart, M., and De La Fuente, L. 2013. *Xylella fastidiosa* differentially accumulates mineral elements in biofilm and planktonic cells. *Plos One* 8:e54936.
- Cruz, L.F., Cobine, P.A., and De La Fuente, L. 2012. Calcium increases *Xylella fastidiosa* surface attachment, biofilm formation, and twitching motility. *Appl Environ Microbiol* 78:1321-1331.
- da Silva, F.R., Vettore, A.L., Kemper, E.L., Leite, A., and Arruda, P. 2001. Fastidian gum: the *Xylella fastidiosa* exopolysaccharide possibly involved in bacterial pathogenicity. *Fems Microbiology Letters* 203:165-171.
- Datnoff, L.E., Elmer, W.H., and Huber, D.M. 2007. Mineral nutrition and plant disease. American Phytopathological Society, St. Paul, Minn.
- Davis, M.J., PURCELL, A.H., and THOMSON, S.V. 1978. Pierce's Disease of Grapevines: Isolation of the Causal Bacterium. *Science* 199:75-77.
- Davis, M.J., French, W.J., and Schaad, N.W. 1981. Axenic culture of the bacteria associated with phony disease of peach and plum leaf scald. *Current Microbiology* 6:309-314.
- De la Fuente, L., Montanes, E., Meng, Y.Z., Li, Y.X., Burr, T.J., Hoch, H.C., and Wu, M.M. 2007. Assessing adhesion forces of type I and type IV pili of *Xylella fastidiosa* bacteria by use of a microfluidic flow chamber. *Appl Environ Microbiol* 73:2690-2696.

- De La Fuente, L., Parker, J.K., Oliver, J.E., Granger, S., Brannen, P.M., van Santen, E., and Cobine, P.A. 2013. The bacterial pathogen *Xylella fastidiosa* affects the leaf ionome of plant hosts during infection. *Plos One* 8.
- Dominguez, D.C. 2004. Calcium signaling in bacteria. *Mol Microbiol* 54:291-297.
- Doyle, J., and Doyle, J.L. 1987. Genomic plant DNA preparation from fresh tissue - CTAB method. *Phytochem. Bull.* 19:11-15.
- Fletcher, M. 1988. Attachment of *Pseudomonas fluorescens* to glass and influence of electrolytes on bacterium substratum separation distance. *Journal of Bacteriology* 170:2027-2030.
- Francis, M., Civerolo, E., and Bruening, G. 2008. Improved bioassay of *Xylella fastidiosa* using *Nicotiana tabacum* cultivar SR1. *Plant Disease* 92:14-20.
- Francis, M., Lin, H., Cabrera-La Rosa, J., Doddapaneni, H., and Civerolo, E.L. 2006. Genome-based PCR primers for specific and sensitive detection and quantification of *Xylella fastidiosa*. *European Journal of Plant Pathology* 115:203-213.
- Fritschi, F.B., Lin, H., and Walker, M.A. 2008. Scanning Electron Microscopy Reveals Different Response Pattern of Four Vitis Genotypes to *Xylella fastidiosa* Infection. *Plant Disease* 92:276-286.
- Gambetta, G.A., Fei, J., Rost, T.L., and Matthews, M.A. 2007. Leaf scorch symptoms are not correlated with bacterial populations during Pierce's disease. *J. Exp. Bot.* 58:4037-4046.
- Goodwin, P.H., DeVay, J.E., and Meredith, C.P. 1988. Physiological responses of *Vitis vinifera* cv. "Chardonnay" to infection by the Pierce's disease bacterium. *Physiological and Molecular Plant Pathology* 32:17-32.
- Hepler, P.K. 2005. Calcium: a central regulator of plant growth and development. *The Plant Cell Online* 17:2142-2155.
- Hopkins, D.L., and Purcell, A.H. 2002. *Xylella fastidiosa*: cause of Pierce's disease of grapevine and other emergent diseases. *Plant Disease* 86:1056-1066.
- Kallstrom, H., Islam, M.S., Berggren, P.O., and Jonsson, A.B. 1998. Cell signaling by the type IV pili of pathogenic *Neisseria*. *Journal of Biological Chemistry* 273:21777-21782.
- Lecourieux, D., Ranjeva, R., and Pugin, A. 2006. Calcium in plant defence-signaling pathways. *New Phytologist* 171:249-269.
- Leite, B., Ishida, M.L., Alves, E., Carrer, H., Pascholati, S.F., and Kitajima, E.W. 2002. Genomics and X-ray microanalysis indicate that Ca²⁺ and thiols mediate the aggregation and adhesion of *Xylella fastidiosa*. *Brazilian Journal of Medical and Biological Research* 35:645-650.
- Li, Y., Hao, G., Galvani, C.D., Meng, Y., De La Fuente, L., Hoch, H.C., and Burr, T.J. 2007. Type I and type IV pili of *Xylella fastidiosa* affect twitching motility, biofilm formation and cell-cell aggregation. *Microbiology* 153:719-726.
- López-Lefebvre, L.R., Rivero, R.M., García, P.C., Sánchez, E., Ruiz, J.M., and Romero, L. 2001. Effect of calcium on mineral nutrient uptake and growth of tobacco. *Journal of the Science of Food and Agriculture* 81:1334-1338.
- Marschner, H. 1995. Mineral nutrition of higher plants. Academic Press, San Diego, California.
- McElrone, A.J., Sherald, J.L., and Forseth, I.N. 2003. Interactive effects of water stress and xylem-limited bacterial infection on the water relations of a host vine. *J. Exp. Bot.* 54:419-430.
- Meng, Y.Z., Li, Y.X., Galvani, C.D., Hao, G.X., Turner, J.N., Burr, T.J., and Hoch, H.C. 2005. Upstream migration of *Xylella fastidiosa* via pilus-driven twitching motility. *Journal of Bacteriology* 187:5560-5567.

- Newman, K.L., R. P. P. Almeida, A. H. Purcell, and S. E. Lindow. 2003. Use of a green fluorescent strain for analysis of *Xyella fastidiosa* colonization of *Vitis vinifera*. *Appl Environ Microbiol* 69:7319-7327.
- Norris, V., Grant, S., Freestone, P., Canvin, J., Sheikh, F.N., Toth, I., Trinei, M., Modha, K., and Norman, R.I. 1996. Calcium signalling in bacteria. *J Bacteriol* 178:3677-3682.
- Onoda, T., Enokizono, J., Kaya, H., Oshima, A., Freestone, P., and Norris, V. 2000. Effects of calcium and calcium chelators on growth and morphology of *Escherichia coli* L-form NC-7. *J Bacteriol* 182:1419-1422.
- Parker, J.K., Havird, J.C., and De La Fuente, L. 2012. Differentiation of *Xylella fastidiosa* strains via multilocus sequence analysis of environmentally mediated genes (MLSA-E). *Appl Environ Microbiol* 78:1385-1396.
- Patrauchan, M.A., Sarkisova, S.A., and Franklin, M.J. 2007. Strain-specific proteome responses of *Pseudomonas aeruginosa* to biofilm-associated growth and to calcium. *Microbiology* 153:3838-3851.
- Patrauchan, M.A., Sarkisova, S., Sauer, K., and Franklin, M.J. 2005. Calcium influences cellular and extracellular product formation during biofilm-associated growth of a marine *Pseudoalteromonas* sp. *Microbiology-Sgm* 151:2885-2897.
- Purcell, A.H., and Hopkins, D.L. 1996. Fastidious xylem-limited bacterial plant pathogens. *Annu Rev Phytopathol* 34:131-151.
- Queiroz-Voltan, R.B., Paradela Filho, O., Carvalho, M.L., and Fahl, J. 1998. Aspectos estruturais de cafeeiro infectado com *Xylella fastidiosa*. *Bragantia* 57.
- Rose, R.K. 2000. The role of calcium in oral streptococcal aggregation and the implications for biofilm formation and retention. *Biochimica Et Biophysica Acta-General Subjects* 1475:76-82.
- Silva-Stenico, M., Pacheco, F., Pereira-Filho, E., Rodrigues, J., Souza, A., Etchegaray, A., Gomes, J., and Tsai, S. 2009. Nutritional deficiency in citrus with symptoms of citrus variegated chlorosis disease. *Brazilian Journal of Biology* 69:859-864.
- Stevenson, J.F., Matthews, M.A., and Ros, T.L. 2004. Grapevine susceptibility to Pierce's disease I: relevance to hydraulic architecture. *American Journal of Enology and Viticulture* 55:228-237.
- Stevenson, J.F., Matthews, M.A., and Rost, T.L. 2005. The developmental anatomy of Pierce's disease symptoms in grapevines: green islands and matchsticks. *Plant Disease* 89:543-548.
- Thorne, E.T., Stevenson, J.F., Rost, T.L., Labavitch, J.M., and Matthews, M.A. 2006. Pierce's disease symptoms: comparison with symptoms of water deficit and the impact of water deficits. *American Journal of Enology and Viticulture* 57:1-11.
- TranVan Nhieu, G., Clair, C., Grompone, G., and Sansonetti, P. 2004. Calcium signalling during cell interactions with bacterial pathogens. *Biology of the cell / under the auspices of the European Cell Biology Organization* 96:93-101.
- Yadeta, K.A., and BP, J.T. 2013. The xylem as battleground for plant hosts and vascular wilt pathogens. *Frontiers in plant science* 4:97.
- Yu, X.-C., and Margolin, W. 1997. Ca²⁺-mediated GTP-dependent dynamic assembly of bacterial cell division protein FtsZ into asters and polymer networks in vitro. *EMBO J* 16:5455-5463.
- Zaini, P.A., De La Fuente, L., Hoch, H.C., and Burr, T.J. 2009. Grapevine xylem sap enhances biofilm development by *Xylella fastidiosa*. *Fems Microbiology Letters* 295:129-134.

Zaini, P.A., Fogaca, A.C., Lupo, F.G.N., Nakaya, H.I., Vencio, R.Z.N., and da Silva, A.M. 2008. The iron stimulon of *Xylella fastidiosa* includes genes for type IV pilus and colicin V-like bacteriocins. Journal of Bacteriology 190:2368-2378.

Chapter 5 Conclusions

In this work, the role of Ca in the pathogenicity of *X. fastidiosa* was established. During the infection process, *X. fastidiosa* requires the expression of different pathogenicity determinants including proteins involved in attachment and biofilm production/maturation that include fimbrial and afimbrial adhesins and exopolysaccharides. In vitro experiments indicated that Ca supplementation increases the amount of biofilm formation. Removal of Ca by intra- and extracellular chelators produced the opposite result, decreasing the amount of biofilm formed. Faster formation of cell aggregates and stronger cell attachment in the presence of Ca was established in microfluidic chambers. It was demonstrated that *X. fastidiosa* cells must be metabolically active for Ca to produce an increase in surface attachment, and effect which seems to require the presence of the type I pili. Ca also enhances the production of exopolysaccharides. Biofilms formed in the presence of Ca were thicker and more compact compared with non-supplemented media. All these findings show that Ca affects all stages of biofilm formation from initial stages that involve cell attachment and aggregation and final stages that involve production of exopolysaccharides for biofilm formation and maturation.

Using twitching motility, *X. fastidiosa* can move against the xylem sap stream in the plant host, allowing systemic vessel colonization, a crucial factor for infection. Ca-supplemented media

increases *X. fastidiosa* motility, thus likely leading to improved infection capabilities. Sequence analysis indicated the presence of a Ca-binding motif in one of the three *pilYI* homologs (PD 1611) encoded in the *X. fastidiosa* genome; this protein is predicted to be a type IV pili tip adhesin. Mutational analyses corroborate the involvement of this specific homolog with the Ca-binding motif in the regulation of twitching. The mechanism of Ca-dependent regulation should be associated with a conformational change in the protein that elicits faster extension and retraction of the pili, resulting in the observed upregulation of *pilT* to retract the pili and, the Ca-binding-motif-containing *pilYI* to extend the pili. Other supporting evidence of the role of interactions between this motif and Ca in the regulation of twitching was found. Sequence analyses and phenotypic characterization correlated the presence of the same motif with an increase in twitching motility in the soil inhabiting bacterium *Rastonia piketti*; likewise other bacterial species with type IV pili lacking this motif did not have a twitching motility response to Ca.

In plant experiments testing the effect of Ca supplementation on the development of *X. fastidiosa* infection indicated that Ca promotes bacterial population growth, which in turn stimulates Ca uptake by the plant. In addition, more severe symptoms were observed in Ca-supplemented plants compared with the non-supplemented plants. Other major changes in the mineral

composition of inoculated plants compared with the non-inoculated plants were observed. In general, alteration in the host mineral status seems to be a virulence strategy of *X. fastidiosa*.