Role of zinc in biofilm production and virulence of *Xylella fastidiosa*
by

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Abstract

Xylella fastidiosa is a Gram-negative plant pathogenic bacterium that exclusively colonizes the xylem vessels of the host plant and the foreguts of sharpshooter insect vectors (Cicadelidae, Cercopidae, Machaerotidae, and Cicadidae). *X. fastidiosa* infects a wide range of hosts, most of them of great economic value, including grape, almond, pecan, blueberry, peach, coffee and citrus trees. Diseases caused by this bacterium are mainly restricted to the Americas, and in the United States, they are restricted to the southern states and California, where the bacterium is endemic. Currently, there is no effective control strategy for *X. fastidiosa.*

X. fastidiosa-infected plants can show several symptoms depending on the host, including marginal leaf scorch and chlorosis, berry mummification, dwarfing, shortened internodes and in extreme cases plant death. These symptoms have been associated with water deficits caused by xylem blockage due to the formation of bacterial biofilms and products of plant defense response such as gums and tyloses.

Xylem sap chemistry has a great impact on the expression of virulence traits by this bacterium, which includes twitching motility, surface and cell-to-cell attachment and biofilm production. Heavy metals, such as Zn have been shown to reduce biofilm production in vitro; however, there are contradictory reports on the role of this metal element during infection. Thus, the objectives of this work were to address the effects of Zn on the physiology and virulence traits of *X. fastidiosa* in vitro and to evaluate its role during disease development. Batch cultures supplemented with Zn showed that this metal reduces growth and biofilm production; however, it induces an increase in the production of exopolysaccharides (EPS). In

microfluidic chambers, under flow condition and with constant bacterial supplementation (closer to conditions inside the host), a dramatic increase in biofilm aggregates was seen when Zn was supplemented to the basal medium. This phenomenon was attributed to the increased EPS production induced by Zn. Additionally; viability analyses suggest that *X. fastidiosa* may be able to enter the viable but non-culturable state *in vitro*, and that Zn can hasten the onset of this state.

The effects of Zn on virulence of *X. fastidiosa* and disease development were assessed by the construction and evaluation of two mutant strains defective in *zur* and *czcD*, two genes involved in Zn homeostasis. When grown in synthetic media both mutants accumulated higher intracellular levels of Zn than the wild type (WT), showing that these genes are involved in the efflux of this metal. The mutant strains were also affected in their abilities to grow in media supplemented with Zn and in xylem sap, compared to the WT. Biofilm production was increased or not affected, in the *zur* and *czcD* mutant; respectively, however this effect was dependent on the concentration of Zn in the medium. EPS production was reduced in both mutant strains only in media supplemented with Zn. Plants inoculated with either *zur* or *czcD* mutants had reduced bacterial populations and reduced symptoms compared to plants inoculated with the WT. Additionally, both mutants failed to produce an extensive alteration to the leaf ionome of inoculated plants, as was seen in plants inoculated with the WT.

Together, these results show that Zn acts as a stress factor that reduces the growth, viability and culturability of *X. fastidiosa,* and suggests that the increased EPS production observed may be a response to counteract the effects of stress. Thus, detoxification of detrimental Zn levels in the host xylem vessels, constitutes an important virulence and survival strategy, which allows a more efficient colonization and alteration of the host physiological status.

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Literature review

Xylella fastidiosa

Xylella fastidiosa is a Gram-negative plant pathogenic bacterium that exclusively inhabits the xylem vessels of the host plant (Hopkins & Purcell, 2002). Cells are rod-shaped, with a radius of 0.25-0.35 μm and a length of 0.9-3.5 μm (Wells J. M., 1987). The bacterium is vectored from plant to plant by xylem sap-feeding insects in the families Cicadellidae, Cercopidae, Machaerotidae, and Cicadidae, commonly known as sharpshooters (Almeida et al., 2005). Inside the host vector, the bacteria attach to the precibarium, the foregut of the insect, from where they are introduced into plants as they detach during insect feeding (Almeida & Purcell, 2006).

X. fastidiosa infects a wide range of hosts, with different subspecies infecting different hosts. *X. fastidiosa* subsp. *fastidiosa* infects grape, alfalfa, almond and maple; *X. fastidiosa* subsp. *multiplex* infects peach, plum, sycamore, elm, blueberry, and almond; *X. fastidiosa* subsp. *pauca* infects citrus and coffee; and *X. fastidiosa* subsp. *sandyi* infects oleander (Schaad et al., 2004, Schuenzel et al., 2005). The most important diseases produced by *X. fastidiosa* are Pierce's disease (PD) of grapevine and citrus variegated chlorosis (CVC). The importance of these diseases lies in the high value of the crops; reductions in yield caused by these diseases represent great economical loss. Other crops such as alfalfa, peach, plum, almond, coffee and many forest trees and landscape plants including oak, elm, and maple are also attacked by the bacterium. The bacterium is mainly restricted to the Americas, although it has been reported in Taiwan and Kosovo (Hopkins & Purcell, 2002). In the United States, it is restricted to the southernmost states, where it is endemic to California and the Gulf Coast

(Hopkins & Purcell, 2002). Currently, there is no effective control for *X. fastidiosa.* Disease management practices are focused on control of the vectors and the use of tolerant or resistant varieties (Hopkins & Purcell, 2002).

Symptom development

X. fastidiosa-infected plants can show several distinct symptoms depending on the host. In grapevines, the distinctive disease symptoms of *X. fastidiosa* infection include: marginal leaf scorch and chlorosis, uneven maturation of the periderm (green islands), premature abscission of leaves leaving the petiole attached to the stem (matchsticks), berry mummification, and in extreme cases plant death (Krivanek et al., 2005). Other hosts can show leaf scorch (coffee, almond, and blueberry), interveinal chlorosis (citrus), dwarfing (alfalfa), or shortened internodes (peach, coffee) (Hopkins & Purcell, 2002, Chang et al., 2009). In many grasses, weeds, and ornamentals, infections develop without any symptoms (Hopkins, 1989).

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) (Krivanek et al., 2005). 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 controversial due to the lack of strong experimental evidence to support it. For instance, studies have shown no correlation between the expression of symptoms and the quantity of bacteria colonizing the xylem (Newman et al., 2003) or the proportion of vessel colonized (Gambetta G. A., 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 in water conductance (Thorne et al., 2006). A recent study showed that *X. fastidiosa* induces water stress in the host; however, this is this is only part of the physiological changes this bacterium produces in the susceptible host (Choi H. K., 2013). These contradictory reports found in the literature point out the limited understanding of the processes leading to symptom appearance.

Pathogenicity mechanisms

Disease development depends on the ability of the bacterium to colonize and multiply in the plant, since only heavily colonized plants show symptoms (Chatterjee et al., 2008a). Cell attachment, movement within the plant, and biofilm formation are considered determinants for plant infection. *X. fastidiosa* encodes various surface-associated proteins that are thought to be important for adhesion to plant surfaces, host colonization, and biofilm formation. The structures involved in these processes are fimbrial and afimbrial adhesins as well as exopolysacharides (EPSs). The most important fimbrial proteins produced by *X. fastidiosa* 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 cell attachment to surfaces and twitching motility. Type I pili are responsible for cell anchorage, and type IV pili extend and retract to assist cell motility (De La Fuente et al., 2007). Afimbrial adhesins are surface proteins that produce projections in the outer membrane. This group of adhesins includes hemmagglutinins, which mediate cell-to-cell adhesion and prevent bacterial movement within the plant (Guilhabert & 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 Alessandra A. et al., 2004, Caserta et al., 2010).

X. fastidiosa moves inside xylem vessels by means of twitching motility, mediated by type IV pili. In this type of surface movement, bacteria extends the type IV pili, its distal end adheres to the surface, and then retraction from the base of the pilus provides the force that pulls the cell forward (Burdman et al., 2011). This type of motility allows *X. fastidiosa* to move inside the plant against the transpiration stream (Meng et al., 2005). However, movement of the bacterium is limited by pit membranes that separate 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. *X. fastidiosa* produces several cell wall-degrading enzymes, particularly a polygalacturonase is necessary for bacterial spread in grape (Roper et al., 2007b). Additionally, an increase in pit membrane pore size in the presence of polygalacturonase and β-glucanase, encoded by *X. fastidiosa*, was detected (Perez-Donoso et al., 2010). This shows that degradation of pit membrane is important for pathogenicity.

Bacteria attached to the xylem vessel walls multiply and form biofilms that, if large enough, can block the vessel completely and are thought to restrain sap flow, although even in symptomatic plants, vessels blocked by bacterial growth are not widespread. Biofilms seem to be required for acquisition by the insect vector (Chatterjee et al., 2008b). Biofilms consists of aggregates of microorganisms attached to each other and to a surface and surrounded by an extracellular matrix; they protect the community of cells from antimicrobials, host defense compounds, dehydration, and to optimize nutrient uptake from the xylem sap flow (Costerton et al., 1995, Danhorn & Fuqua, 2007). Biofilm formation can be divided into different stages: initial attachment of cells to the surface, production of EPS resulting in more firmly "irreversible" attachment, maturation and development of biofilm architecture, and dispersion of single cells from the biofilm (Stoodley et al., 2002). The synthesis of EPS is essential for biofilm production, maturation and virulence in many plant pathogenic bacteria (Denny,

1995). Recently, it was demonstrated that *X. fastidiosa* EPS production is essential for pathogenicity and insect transmission (Killiny et al., 2013). In *X. fastidiosa*, as well as in other bacteria, production of extracellular DNA enhances biofilm growth (Cheng, 2010).

As in other species of bacteria, quorum sensing plays a key role in pathogenicity. *X. fastidiosa* harbors a cell-to-cell signaling system mediated by a fatty acid molecule called diffusible signal factor (DSF), encoded by the *rfp* cluster of genes which regulates bacterial aggregation, attachment, biofilm formation, insect transmission, and virulence (Chatterjee et al., 2008a). The RpfF protein synthesizes DSF, which is exported to the extracellular medium and accumulated when cell densities are high. DSF is sensed by a two component system, RpfC/RpfG, which regulates the expression several traits, repressing the expression of polygalacturonase and type IV pili and promoting the expression of adhesins and EPS. Thus, at low population densities, bacterial movement and plant colonization is favored whereas at high cell densities, biofilm formation and insect acquisition are promoted (Chatterjee et al., 2008a).

Influence of mineral elements on the virulence of *Xylella fastidiosa*

Since the presence of *X. fastidiosa* within the plant is restricted to the xylem vessels, the bacterium must be able to obtain all required nutrients from the xylem sap and the vessel walls. Thus, it can be hypothesized that xylem sap chemistry has an important effect on bacterial growth and virulence. In fact, xylem fluid from PD-resistant *Vitis rotundifolia* maintained *X. fastidiosa* in a planktonic state (not attached to a surface), whereas the bacterium was more likely to form aggregates when incubated in PD-susceptible *V. vinifera* cultivars (Leite B, 2004). Amendment of synthetic media with xylem sap from the susceptible

V. vinifera ‘Chardonnay’ promotes surface attachment and biofilm production, which have a very compact architecture (Zaini et al., 2009).

Chemical analysis of xylem sap has shown that planktonic growth correlates with the levels of certain amino acids as well as copper (Cu), magnesium (Mg), phosphorus (P), and zinc (Zn). Also, addition of calcium (Ca) and magnesium together to extracted xylem sap used as an in vitro culture medium promotes cell aggregation (Andersen et al., 2007). In fact Ca, added to synthetic media, was shown to promote several virulence traits which are necessary to promote colonization of the host plant, including twitching motility, attachment to surfaces, and biofilm formation (Cruz et al., 2012). Ca, together with Mg, has been proposed to act as bridge between the negatively charged surfaces of the xylem vessels and the bacterial cells to promote cell attachment and aggregation (Leite et al., 2002). Iron has also been implicated in the regulation of *X. fastidiosa* virulence. This metal promotes the expression of genes involved the synthesis of type IV and type I pili, as well as the synthesis of bacteriocins, which act in the competition against other bacteria inside the xylem (Zaini et al., 2008).

Chemical analysis of biofilm-forming versus planktonic *X. fastidiosa* cells has shown a markedly different metal composition. Biofilm cells accumulate high levels of Cu, Manganese (Mn) and Zn compared to planktonic ones. In vitro, Cu promotes attachment and aggregation at low levels, whereas at higher concentrations it prevents growth. Mn promotes cell-cell aggregation and biofilm production, whereas Zn reduces planktonic and biofilm growth (Cobine et al., 2013). A Zn-protease is induced in *X. fastidiosa* in planta which utilizes free amino acids in the xylem as nitrogen and carbon (Purcino et al., 2007). Zn-metalloproteases are also upregulated during biofilm formation (Silva et al., 2011).

Additionally, Zn increases the activity of extracellular proteases produced by this bacterium (Fedatto et al., 2006).

Role of zinc in living organisms

Zinc is the most abundant non-redox transition metal in biological systems, and in aqueous solutions it exists as a divalent cation that can form many soluble salts. The solubility of Zn compounds tends to increase as the pH of the medium decreases (Marschner, 1995). Due to its complete *d*-shell of electrons Zn^{2+} is redox stable under biological conditions, in contrast to other biologically important metals such as Fe, Cu, and Mn. Zinc is a Lewis acid, which allows it to form strong bonds with S, N, and O (Broadley et al., 2007). These properties largely determine the functions of Zn in biological systems. Zinc has the primary function of assuring correct macromolecular folding and stability; however, despite these essential roles, Zn can be toxic at high concentrations.

The amount of Zn-binding proteins in bacteria has a direct relationship to the optimal growth temperature of the organism (Andreini et al., 2006). Thus, Zn is believed to provide macromolecules with the additional stability required at higher temperatures. In proteins, Zn is flexible in terms of coordination chemistry. It can be bound by four to six ligands including cysteine, histidine, glutamate, aspartate, and water (Mitra, 2007). Zn-binding proteins tend to perform different functions in eukaryotes and prokaryotes. Most eukaryotic Zn-binding proteins are Zn finger proteins (DNA-binding) or PHD domain-containing proteins (reminiscent of Zn finger domain, involved in protein-protein interactions), whereas in prokaryotes, most Zn-binding proteins are metallophosphatases, peptidases, and lactamases (Andreini et al., 2006). Additionally, Zn can affect the integrity of cell membranes by binding

strongly to sulfhydryl groups and increasing their stability. Accordingly, Zn can prevent lipid peroxidation (Chvapil, 1973, Goel et al., 2005).

Role of zinc in bacterial metabolism

Since Zn has a widespread role in protein stabilization and catalysis, this metal is important for a broad range of cellular processes in which Zn-binding proteins are involved. The most noteworthy of these processes are DNA and RNA synthesis as well as protein synthesis and cleavage. Accordingly, it has been estimated that between 12-50% of the Zn pool of the cell is employed by the transcriptional and translational machinery (Finney & O'Halloran, 2003). Zinc binds DNA nucleotide bases, contributing to the stability of the DNA double-helix (Chvapil, 1973). Even more importantly, Zn is a required cofactor of some bacterial and eukaryotic DNA polymerases. In fact, *Salmonella typhimurium* grown in Zn-supplemented media shows a higher DNA polymerase activity that the non-supplemented control (Wu & Wu, 1987). Bacterial and eukaryotic RNA polymerases also contain Zn as a cofactor (Auld et al., 1976, Halling et al., 1977). In prokaryotes, this metal not only stabilizes the RNA polymerase but also regulates promoter recognition, as well as recognition and orientation of the initiating nucleotide in the RNA synthesis (Wu & Wu, 1987).

Protein synthesis also relies on Zn availability, since it is a component of many aminocyl-tRNA synthetases and ribosomes. A Zn-binding domain is involved in recognition of the acceptor stem of the tRNAs (Ibba & Soll, 2000); whereas in ribosomal proteins, a cysteine rich ribbon that is believed to bind Zn to stabilize the ribosomes at high temperatures (Makarova et al., 2001). Zinc-metalloproteases, widespread among bacteria, are also important for protein cleavage. These enzymes catalyze the hydrolysis of peptide bonds in terminal or internal amino-acidic residues of polypeptides. In these metalloproteases, a Zn

atom is localized at the catalytic site and is coordinated by the consensus motif HEXXH (Hase & Finkelstein, 1993, Miyoshi & Shinoda, 2000).

Zinc homeostasis in bacteria

The importance of Zn for bacteria is evident from the energy spent by the cell to accumulate this metal from the surrounding media, up to three orders of magnitude higher than standard artificial media, reaching total intracellular concentrations of 0.1 mM (Outten & O'Halloran, 2001). Nevertheless, accumulation of Zn beyond optimal concentrations leads to toxicity, since this metal can inhibit the electron transport chain presumably by replacing Fe in Fe-S containing proteins (Chvapil, 1973). At high concentrations, Zn can also interfere with the metabolism of Mg and Mn, as some Mg and Mn uptake systems can transport Zn as well (Blencowe & Morby, 2003). In *S. pneumoniae*, high Zn concentrations can inhibit Mn uptake, leading to Mn starvation (McDevitt et al., 2011).

Zinc uptake can be mediated by high or low affinity transport systems. The best characterized high affinity Zn uptake system in bacteria is ZnuABC, an ABC transport system pumped by ATP hydrolysis. Some Gram-negative bacteria also possess an outer membrane protein named ZnuD which associates with TonB and transports Zn and heme. Both the *znuABC* operon and *znuD*, have their expression repressed when Zn is present at micromolar amounts in the medium (Patzer & Hantke, 1998, Stork et al., 2010). The high affinity uptake systems are under transcriptional regulation of the zinc uptake regulator (Zur) (Patzer & Hantke, 1998, Stork et al., 2010).

Zur, which is crucial for Zn homeostasis and ubiquitous among bacteria, belongs to the Fur (ferric uptake regulator) family exhibiting a (helix-turn-helix) DNA binding domain at the N-terminus and a dimerization domain at the C-terminus (Patzer & Hantke, 2000, Hantke,

2002). Zur activity is regulated by binding of Zn^{2+}. At elevated concentrations of Zn^{2+}, Zur binds to specific promoter sequences of target genes, altering their expression, for example repressing the transcription of *znuABC* (Patzer & Hantke, 2000). In some cases, the expression of Zn uptake systems can also be regulated by Fur (Garrido et al., 2003).

Low affinity Zn uptake can also be mediated by members of the ZIP family or by proteins of the NRAMP family (Natural Resistance Associated Macrophage Protein) although these proteins mainly transport Mn. In addition, some Mg transporters mediate Zn uptake as well (Blencowe & Morby, 2003).

Once in the cytosol, the concentration of Zn is regulated by ribosomal proteins. Ribosomal proteins that contain the aforementioned Zn-binding ribbon tend to be duplicated in bacterial genomes, but their paralogs lack the Zn-binding ribbon. Under Zn-replete conditions Zur represses the expression of the paralogs lacking the Zn-binding motif; whereas in Zn depleted conditions, the non-Zn-ribbon paralogs are expressed and partially replace the corresponding Zn-binding proteins, making Zn available for other processes (Panina et al., 2003, Shin et al., 2007).

Resistance to high Zn concentration is mediated by metal sequestration (Choudhury & Srivastava, 2001, Blindauer et al., 2002), detoxification of the medium (Choudhury & Srivastava, 2001), and efflux systems. The best characterized efflux systems include P-type ATPases, Cation Diffusion Facilitators (CDF) and Heavy Metal Efflux Resistance-Nodulation-Cell Division efflux systems (HME-RND). The Zn-CPx-type ATPase family mediates efflux of Zn^{2+}, Cd^{2+} and Pb^{2+} from the cytoplasm to the periplasm. ZntA, a Zn-CPx-type ATPase from *Escherichia coli*, is regulated at the transcriptional level by ZntR (a MerR-

like transcriptional regulator) which acts as a direct Zn^{2+} sensor and activates the transcription of *zntA* at Zn concentrations of 100 μM or higher (Blencowe & Morby, 2003). CDF proteins localize at the cell membrane and pump divalent cations (preferentially Zn^{2+}) to the periplasm or cell exterior driven by H^+ or K^+ gradients (Nies, 2003).

In the plant pathogen *Xanthomonas campestris*, the expression of *czcD* (CDF family) is regulated by Zur, which binds to the *czcD* promoter and activates its transcription when the Zn concentration is high. Of note, the promoter sequence recognized by Zur during *czcD* transcriptional activation is different from the one involved in Zur-mediated repression of *znuABC* (Huang & Tang, 2008). Interestingly, bacteria relying exclusively on CDF proteins for Zn efflux are adapted to anaerobic or microaerobic environments (Nies, 2003).

The HME-RND family of proteins transports heavy metals through the inner membrane, periplasm, and outer membrane. The energy required for this process is provided by the proton gradient. In *Ralstonia metallidurans*, the HME-RND system consists of a chemosmotic cation/H^+ antiporter, a membrane fusion protein that may form a periplasmic funnel, and an outer membrane protein; and also transports Cd^{2+}, Zn^{2+}, and Co^{2+} (Blencowe & Morby, 2003, Nies, 2003). HME-RND have a more restricted distribution in prokaryotes than Zn-CPx-type ATPase and CDF proteins, and the presence of HME-RND correlates with high resistance to Zn, Cd and Co (Nies, 2003).

Finally, a very remarkable aspect of Zn homeostasis in bacteria is the fact that there are no persistent free Zn ions (non-ligand-bound) in the cell under non-stress conditions. *In vitro*, high-affinity Zn uptake systems are repressed at the same range of free Zn concentrations at

which Zn efflux systems are activated (around 0.5 fM); if the volume of the cell is considered, this is equivalent to less than one free cytosolic Zn atom per cell (Outten & O'Halloran, 2001).

Role of zinc in bacterial virulence

In order to establish a successful infection, pathogens must precisely regulate their virulence traits in response to the host environment. Due to the importance of the cell processes in which Zn is involved, alterations in its homeostasis lead to alterations in virulence. In the next section, the influence of Zn on virulence traits of plant pathogenic bacteria will be reviewed in the context of animal pathogens, which are better characterized.

Zinc transporters as virulence traits

When colonizing host tissues, nutrient acquisition can have a major influence on the success of an infection. Animal pathogens face low levels of available nutrients in host tissues; furthermore nutrient deprivation is a known strategy of mammalian innate immunity (Hood & Skaar, 2012). Zn transporters have been established as factors contributing to host colonization in several animal pathogens (Lewis et al., 1999, Yang et al., 2006). In uropathogenic *E. coli*, mutants of *znuABC* and, to a lesser extent, *zupT* (ZIP family) mutants are defective in virulence. These mutants develop lower populations than with the wild-type parental strain in the kidneys of inoculated mice. The reduction in virulence seems to be due to lower oxidative stress tolerance and reduced motility (see 4.5.2). Proteins involved in both processes contain Zn in their structure (Zn-Cu superoxide dismutase and flagella proteins) (Sabri et al., 2009).

High affinity Zn transport systems also seem to be crucial for intracellular pathogens. In *S. enterica*, the expression of *znuA* (member of the *znu*ABC operon) is up-regulated during

intracellular and spleen infection. Additionally, a *znuA* mutant is reduced in virulence, reaching lower populations inside epithelial host cells compared to the wild-type (Ammendola et al., 2007). Among plant associated bacteria, only *znuABC* from *Sinorhizobium meliloti* and '*Candidatus* Liberibacter asiaticus' have been experimentally characterized to date. *znuABC* from *S. meliloti* is Zn-responsive and can complement *znuABC* mutants of *E. coli*. Fertilization with Zn has been shown to increase nodulation of *S. meliloti* in alfalfa (Grewal, 2001). On the other hand, '*Ca.* Liberibacter asiaticus' , unlike the majority of bacteria, has two copies of the *znu*ABC operon in its chromosome and lacks *zur* or *fur* homologs in its genome, suggesting that in this species *znu*ABC could be expressed constitutively, possibly due to constantly low Zn availability inside host cells. On the contrary, *S. meliloti* presents a Zur homolog, which is in agreement with its more complex lifestyle, inhabiting both soil and host plant cells, thus requiring a more elaborate regulation of this Zn transporter (Vahling-Armstrong et al., 2012).

Effects on bacterial motility

Flagella are required for movement in liquid media and are also implied to be involved in adhesion in several bacterial species (Moens & Vanderleyden, 1996). Zinc is known to induce flagellin synthesis in *E. coli* (Guzzo et al., 1991) and exposure of *E. coli* to TPEN (N,N,N′,N′-Tetrakis(2-pyridylmethyl)ethylenediamine, a Zn chelator) down-regulates the expression of several genes involved in flagella synthesis (Guzzo et al., 1991). Additionally, a *znuABC-zupT* double mutant of *E. coli* exhibits reduced swimming compared to the wild-type. This phenotype can be partially restored by addition of Zn to the medium (Sabri et al., 2009).

Proteus mirabilis exhibits a type of motility called swarming motility, in which cells elongate ten times or more compared to their normal length, become hyperflagellated and migrate in

mass (Rather, 2005). In this species, when Zn uptake is prevented, either by mutation of *znuC* or addition of the Zn-chelator TPEN to the medium, reduced flagellin expression and swarming motility is observed, cells still elongate although not to the wild-type levels (Nielubowicz et al., 2010). Interestingly, mutation of *ppaA* (a Zn-CPx-type ATPase) produces a very similar phenotype – reduced flagellin expression and swarming motility; although, in this mutant, cells are markedly reduced in cell elongation. Additionally, in wild-type cells, the expression of *ppaA* and flagellar genes peaks during swarming cell differentiation (Lai et al., 1998). Taken together these observations indicate that rather than a high Zn level, adequate Zn homeostasis is required for flagellar-mediated motility. Though illustrated in bacterial pathogens of animals, information on the effect of Zn on bacterial motility in bacterial plant pathogens is largely unavailable.

Impact on type III secretion system

The T3SS is a needle-like structure that spans the bacterial membrane and cell wall and pierces the host cell wall and membrane to deliver proteins involved in virulence (called Type 3 effectors). This secretion system is widespread in Gram-negative bacteria and is known to be a pathogenicity factor (Galan & Wolf-Watz, 2006). Recognitions of Type 3 effectors by a resistant host lead to localized cell death, known as hypersensitive response (HR) (Segonzac & Zipfel, 2011).

In the plant pathogen *X. campestris* pv. *campestris*, the expression of a group of genes that code for the core components of the T3SS have been shown to be under the control of Zur. In this strain, a *zur* mutation leads to a delayed HR when inoculated in a non-host plant. Zur indirectly activates the transcription of *hrpX*, a regulator of the T3SS structural genes, which in turn activates the transcription of the core components of the T3SS (Huang et al., 2009). To

date, there are no other reports of regulation of the T3SS by Zn or Zn-binding proteins in plant pathogenic bacteria.

Effects on toxin and protease production

Toxins are a well-recognized type of virulence factor in *Pseudomonas syringae* (Nomura et al., 2005). Tabtoxin-β-lactam is a toxin secreted in an inactive form (tabtoxin), which inhibits ammonia detoxification and causes chlorosis in the plant host. In the bacterial periplasm, tabtoxin is cleaved into tabtoxin-β-lactam by a Zn-metalloprotease. Some *P. syringae* strains exhibit increased tabtoxin-β-lactam production when Zn is added to the growth medium (Durbin & Uchytil, 1985, Bender, 1999).

Metalloproteases involved in virulence have been found in many animal and plant pathogenic bacteria, including *Streptococcus* spp., *Lysteria monocytogenes*, *P. carotovorum* and *X. campestris* among others (Hase & Finkelstein, 1993). *B. cenocepacia*, a bacterium that can infect both plants and animals, presents at least two Zn-metalloproteases required for full virulence in an animal host (Kooi et al., 2006). In *P. aeruginosa*, Zn as well as Ca, stimulates the production of elastase (a Zn-metalloprotease) (Brumlik & Storey, 1992, Olson & Ohman, 1992).

Role in cell attachment

Attachment is crucial for pathogens that colonize solid surfaces such as the mammalian urinary tract, lung tissue or plant surfaces (Rodríguez-Navarro et al., 2007, Reed & Williams Jr, 1978). In *Streptococcus* spp., Zn seems to have a regulatory role in attachment, presumably influenced by the low concentrations of Zn that can be reached in the respiratory mucosal tissue that these pathogens colonize (Hood & Skaar, 2012). In *Streptococcus pyogenes* and *S. pneumoniae* the expression of laminin (an adhesion protein) is repressed

under high concentrations of Zn, and this is mediated by AdcR, the Zn transcriptional regulator in this bacterial group (Elsner et al., 2002, Panina et al., 2003, Shafeeq et al., 2011). There are no reports of Zn affecting adhesins production or activity in plant pathogenic bacteria.

Effects on exopolysaccharide production

Many bacteria produce EPS that can remain attached to the cell surface or be released into the medium. EPS is the major extracellular constituent of biofilms, and biofilm production is a well-documented virulence-related phenotype in many plant pathogenic bacteria (Denny, 1995).

In *X. campestris* pv. *campestris, zur* mutants have reduced EPS production and virulence (Tang et al., 2005). In *Xanthomonas oryza*e pv. *oryzae,* a *zur* mutant has the same aforementioned phenotype, and, additionally, it is more sensitive to oxidative stress (Yang et al., 2007). The exact mechanism is not known, but the reduced EPS production could leave the cells more exposed and thus more sensitive to reactive oxygen species (Yang et al., 2007). How Zur exerts its effect on EPS production has not been established; however, it is worth noticing that in *X. campestris* the activity of phosphomannose isomerase, an enzyme involved in EPS biosynthesis, is enhanced by addition of Zn and this metal is known to be a component of phosphomannose isomerase in *Saccharomyces cerevisiae* (Papoutsopoulou & Kyriakidis, 1997). In this Zn-mediated regulation of phosphomannose isomerase activity, it can be speculated that Zur binds to a yet unknown sequence in the promoter of the phosphomannose isomerase coding gene and induce its expression.

Zinc metabolism in *Xylella fastidiosa*

According to the annotation of the *X. fastidiosa* genome, there are approximately 30 genes whose products interact with Zn. The vast majority of them are involved in protein metabolism (proteases, amynoacyl t-RNA synthetases, ribosomal proteins, and chaperones). Other genes, of less abundance, that code for proteins with Zn binding sites in *X. fastidiosa* are involved in DNA replication, synthesis of LPS and polysaccharides.

Zn homeostatic genes include the aforementioned *zur* and two classes of Zn resistance systems, *czcD* (CDF family) and *czcABC* (HMR-RND family). Intriguingly, there are no obvious Zn importers coded by the genome of *X. fastidiosa*. Even BLAST searches using known Zn importers from other species return matches of very low identity or no matches at all (De la Fuente et al. *in press*) . This information suggests that this bacterium may import Zn trough non-specific transporters and that low Zn availability is not usually faced in nature.

Hypothesis:

With the background knowledge presented above, it was decided to test the Hypothesis that zinc, being transported through the xylem to be used as a nutrient by the plant, affects the growth, physiology and virulence of *Xylella fastidiosa.*

General Objective

Evaluate the effects of Zinc on growth, biofilm formation and virulence of *Xylella fastidiosa*

Specific Objectives

1. Evaluate the effect of Zinc on the physiology and virulence traits of *Xylella fastidiosa in vitro.*
2. Construct mutants affected in zinc homeostasis and evaluate the effect of the mutations on the virulence of *Xylella fastidiosa* in a model host.

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Chapter II

Xylella fastidiosa response to zinc: decreased culturability, increased exopolysaccharide production, and resilient biofilms under flow conditions*

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Running title: *Xylella fastidiosa* response to zinc

Abstract

The bacterial plant pathogen *Xylella fastidiosa* produces biofilm that accumulates in the host xylem vessels, affecting disease development in various crops and bacterial acquisition by insect vectors. Biofilms are sensitive to the chemical composition of the environment, and mineral elements being transported in the xylem are of special interest for this pathosystem. Here, *X. fastidiosa* liquid cultures were supplemented with zinc and compared with non-amended cultures for determining the effect of Zn on growth, biofilm, and exopolysaccharide (EPS) production in batch and flow culture conditions. Results show that Zn reduces growth and biofilm production in both conditions. However, in microfluidic chambers under liquid flow and with constant bacterial supplementation (closer to conditions inside the host), a dramatic increase in biofilm aggregates was seen in the Zn-amended medium. Biofilms formed under these conditions were strongly attached to surfaces and were not removed by media flow. This phenomenon was correlated with increased EPS production in stationary phase cells grown under high Zn concentrations. Zn did not cause greater adhesion to surfaces by individual cells. Additionally viability analyses suggest that *X. fastidiosa* may be able to enter the viable but non-culturable state *in vitro*, and Zn can hasten the onset of this state. Together, these findings suggest that Zn can act as a stress factor with pleiotropic effects on *X. fastidiosa* and indicate that, although Zn could be used as a bactericide treatment, it could trigger the undesired effect of stronger biofilm formation upon re-inoculation events.

Keywords: *Xylella fastidiosa*, zinc, biofilm, microfluidics, EPS, culturability

Introduction

The bacterial plant pathogen *Xylella fastidiosa* is a member of the Xanthomonadaceae (γ-Proteobacteria) that colonizes only the xylem of its host plants and the foregut of xylem-feeding sharpshooter insect vectors (Cicadellidae) (Hopkins & Purcell, 2002, Chatterjee et al., 2008a). *X. fastidiosa* is associated with a wide range of plant diseases causing great economic losses, including Pierce's disease of grapevine (PD), citrus variegated chlorosis (CVC), almond leaf scorch (Hopkins & Purcell, 2002), and bacterial leaf scorch of blueberry (Chang et al., 2009). Also, many grasses and weeds can be colonized asymptomatically by *X. fastidiosa* (Hopkins, 1989).

Typical symptoms of *X. fastidiosa* infection appear at the end of the summer and consist of leaf chlorosis, marginal scorching, and/or dwarfing, depending on the host, and are associated with systemic blockage of xylem vessels (Hopkins & Purcell, 2002). Symptoms have been attributed to prolonged water stress caused by *X. fastidiosa* growth and biofilm formation in the xylem vessels (Goodwin et al., 1988). Recent evidence has shown that, though *X. fastidiosa* indeed induces water stress, this is only part of the physiological changes this bacterium produces in the susceptible host (Choi H. K., 2013). In addition to changes in water balance, *X. fastidiosa* infection also alters nutrient uptake in infected plant hosts (Choi H. K., 2013, De La Fuente et al., 2013). Changes in the levels of Ca, K, P, and other minerals have been found in leaves of diseased plants compared to control plants (Goodwin et al., 1988, Andersen & French, 1987, Silva-Stenico et al., 2009, De La Fuente et al., 2013). Furthermore,

interveinal chlorosis produced by *X. fastidiosa* infection resembles Fe or Zn deficiency in grape and citrus, respectively (Queiroz-Voltan, 1998); however, the effects of *X. fastidiosa* infection on Zn levels in plant tissues are not well-defined. Some authors have reported Zn deficiencies in *X. fastidiosa*-infected citrus leaves (Malavolta, 1991), whereas others have reported increased Zn levels in chlorotic areas of infected citrus leaves (Silva-Stenico et al., 2009). Moreover, recent studies by our group show that infection with *X. fastidiosa* does not cause significant changes in the levels of Zn in hosts (De La Fuente et al., 2013). Likewise, *in vitro* studies of the effect of Zn on *X. fastidiosa* have produced contradictory results: while supplementation of synthetic medium with Zn reduces the growth of *X. fastidiosa* (Cruz et al., 2012, Cobine et al., 2013), Zn concentrations in xylem vessels have been positively (Andersen et al., 2007) or negatively (Malavolta, 2005, Brady, 2010) correlated with growth of *X. fastidiosa*. Application of Zn drenches to grapevines was shown to decrease *X. fastidiosa* populations, but the effects on disease development were inconsistent (Brady, 2010). Nonetheless, a Zn-protease is induced during *X. fastidiosa* infection in citrus that allows for utilization of amino acids present in the xylem as nitrogen and carbon sources, which suggests that the concentration of Zn in the environment may affect the survival and virulence of *X. fastidiosa* (Purcino et al., 2007).

The well-characterized virulence factors that help *X. fastidiosa* efficiently colonize its hosts and develop disease includes twitching motility and cell wall-degrading enzymes (Chatterjee et al., 2008a). Additionally, production of adhesins and exopolysaccharides (EPS) contributes to surface attachment, cell-to-cell aggregation, biofilm formation inside xylem vessels, and insect transmission (Chatterjee et al., 2008a). Biofilms are structured, surface-associated communities of bacteria surrounded by an extracellular matrix that protects bacteria from harsh environmental conditions (Costerton et al., 1995, Branda et al., 2005). The biofilm

matrix can contain EPS, proteins, and/or nucleic acids (Branda et al., 2005), and during infection, EPS protects bacteria from coming into contact with toxic molecules and can induce symptoms such as wilt in the host, as in *Ralstonia solanacearum* or *Erwinia amylovora* infections (Denny, 1995). In *X. fastidiosa*, it was demonstrated recently that EPS production is fundamental for biofilm production, virulence, and insect transmission (Killiny et al., 2013) and that *X. fastidiosa* biofilms accumulate high levels of Zn and other heavy metals (Cobine et al., 2013). Also, in the closely related *Xanthomonas* spp., mutants affected in Zn homoeostasis are compromised in EPS production and virulence (Tang et al., 2005, Yang et al., 2007).

The objective of this study was to define the effects of sub-lethal concentrations of Zn on *X. fastidiosa* virulence traits *in vitro*. Two different culture conditions were used: i) batch systems (test tubes, conicals, and Erlenmeyer flasks) with no replenishing of nutrients, and ii) flow conditions (microfluidic chambers) with multiple inoculation events and continual nutrient flow, mimicking the infection process inside xylem vessels. Results show that Zn inhibits bacterial growth and biofilm production in batch cultures. Moreover, it is shown here that *X. fastidiosa* cultures can enter a putative viable but non-culturable (VBNC) state that is hastened by Zn. Increased production of EPS when *X. fastidiosa* was grown in a Zn-amended medium was also observed, which may contribute to the formation of biofilm structures resistant to shear force caused by flow conditions. The findings presented here help explain previous contradictory reports on the effects of Zn on this plant pathogen.

Materials and Methods

Strains and inoculation procedures

All experiments were carried out using *X. fastidiosa* strain Temecula (Wells J. M., 1987) unless otherwise stated. For evaluation of biofilm structure, *X. fastidiosa* strain KLN59.3, which expresses green fluorescent protein (GFP), was used (Newman et al., 2003). For inoculation of liquid cultures, cells grown on PW (Davis et al., 1980) agar for 7 days were resuspended in 1 ml of PD2 (Davis et al., 1981) and 10 µl were used to inoculate test tubes. Fifty ml flasks were inoculated to an initial $O.D._{600nm} = 0.01$. In the case of microfluidic chambers, cell suspensions ($O.D._{600nm} = 0.1$) were collected with a 1 ml syringe and injected in the chambers with a syringe pump (Pico Plus; Harvard Apparatus, Holliston, MA). Stocks of *X. fastidiosa* cultures were stored in PW (Davis et al., 1980) broth plus 20% glycerol at -80°C.

Bacterial growth under different Zn concentrations

X. fastidiosa was grown in glass test tubes containing 3 ml of PD2 or PD2 amended with different amounts of $ZnSO_4$ ranging from 25 to 2000 µM. Concentrations assayed were 25, 50, 75, 100, 200, 300, 400, 500, 600, 700, 1000, and 2000 µM, with three replicate tubes per concentration. Inoculated tubes were incubated at 28°C and 150 rpm for 10 days. Four independent experiments were performed. Planktonic growth was measured by reading the $O.D._{600\ nm}$ of one ml of liquid culture that was thoroughly mixed using a micropipette prior to measurement. Biofilm growth was evaluated by crystal violet assay according to Zaini *et al.* (Zaini et al., 2009). Briefly, after planktonic growth was recorded, the remaining broth was removed and the tubes were gently rinsed twice with distilled water. Biofilms were stained

with 4 ml of 0.1% crystal violet for 20 min. Tubes were rinsed with distilled water as above, 4 ml of 6:4 (vol/vol) acetone/ethanol were added, and the tubes were agitated at 150 rpm for 5 min. The $O.D._{600\ nm}$ of the resulting acetone/ethanol solution was measured.

Evaluation of total, viable, and culturable cells

X. fastidiosa was grown in 250 ml flasks containing 50 ml of PD2 or PD2 amended with 400 µM $ZnSO_4$ and incubated at 28°C and 150 rpm for 10 days. Each flask contained three glass slides attached to its bottom by a polydimethylsiloxane (PDMS, Sylgard184, Dow Corning, Midland, MI) base to allow for repetitive sampling of the biofilm. At each time point (5, 7 and 10 days post inoculation-dpi), one glass slide was removed from the flask, the biofilm was aseptically scraped off, resuspended in 1.5 ml PD2, and thoroughly homogenized with a micropipette. Two hundred microliters of the suspension were used directly for DNA extraction (three replicates) using a modified CTAB protocol (Doyle J., 1987), 200 µl were treated with ethidium moanoazide (EMA) (Nocker & Camper, 2006) and subsequently used for DNA extraction (three replicates), and 100 µl of the appropriate suspension dilution were plated on PW agar plates (two replicates), prior to a repeated homogenization step, and incubated at 28°C for 2 weeks for enumeration of colony-forming units (CFU). For the planktonic phase, a 1.5 ml aliquot was collected and treated in the same manner as the biofilm at each time point (3, 5, 7 and 10 dpi). Three biological replicates were used per treatment and two independent experiments were performed.

The number of total ("total cells ml^{-1}") and viable ("viable cells ml^{-1}") *X. fastidiosa* cells in each sample was determined by quantitative polymerase chain reaction (qPCR) of the non-EMA-treated DNA samples and EMA-treated DNA samples, respectively. qPCRs were

conducted using previously described primers and TaqMan® probe set (Francis et al., 2006) (Table 1). Briefly, reactions (20 µl) consisted of 1X ABsolute Blue QPCR ROX mix (ABgene-Thermo Fisher Scientific, Surrey, United Kingdom), 0.4 µl TaqMan probe (labeled 5'-6-carboxyfluorescein, 3'-Black Hole Quencher-1®, 6-FAM™-BHQ-1), 0.2 µl each primer, and 1 µl DNA. Samples were amplified using an ABI 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA) with the following cycling parameters: 95°C for 1 min, followed by 40 cycles of 95°C for 15 s and 60° C for 1 min.

As previously described (De La Fuente et al., 2013), a four-point standard curve was amplified alongside each set of qPCR reactions for sample quantification. Briefly, qPCR standards were produced by serially-diluting DNA extracted from a known quantity of *X. fastidiosa* colony-forming units (CFU). *X. fastidiosa* cells grown on PW agar for seven days were suspended in PD2, thoroughly de-aggregated with a micropipette, and DNA was extracted from a known volume of this suspension to provide DNA for the qPCR standards. The remainder of this bacterial suspension was serially-diluted, spread-plated, and incubated until CFUs were visible. CFUs were enumerated, and CFU/ml in the original bacterial suspension was calculated for determination of CFU/ml in qPCR standards.

The number of culturable *X. fastidiosa* cells ("culturable cells ml^{-1}") in each sample was determined from CFU counts on PW agar plates. For result comparisons, data from the three methods are referred to as number of "cells" and concentrations were expressed by volume (ml).

Biofilm growth in microfluidic chambers

Microfluidic chamber fabrication was performed as previously described (De La Fuente et al., 2007). The microfluidic chambers used contain two parallel channels molded into PDMS that allow for observation of different conditions simultaneously, each with a separate entry channel used for bacterial inoculation (De La Fuente et al., 2007). The channels were filled with PD2 or PD2 amended with 400 μM $ZnSO_4$ using an automated syringe pump (Pico Plus; Harvard Apparatus, Holliston, MA), and the flow rate was maintained at 0.25 μl/min. *X. fastidiosa* cell suspensions were injected overnight at 0.1 μl/min. After cell aggregates were visible in the PD2 channel (5-7 days), the chambers where re-inoculated in both channels by injecting additional cell suspension overnight as described above. The newly-introduced inoculum was obtained from stationary-phase cells that were incubated at room temperature over 5-7 days in a 1-ml syringe connected by tubing to the channels. Microfluidic chambers were then monitored for an extra two to three days after reinoculation. The microfluidic chambers were mounted onto a Nikon Eclipse Ti inverted microscope (Nikon, Melville, NY) and observed with a 40X objective using phase-contrast or Nomarski differential interference contrast (DIC) optics. Cell aggregation over time was recorded using time-lapse video imaging microscopy. Image acquisition was done using a Nikon DS-Q1 digital camera (Nikon, Melville, NY) controlled by NIS-Elements software version 3.0 (Nikon, Melville, NY). Four independent experiments were performed.

Evaluation of surface attachment

Two surface attachment parameters were analyzed: adhesion force and percentage of attached cells. Adhesion force was evaluated inside microfluidic chambers in PD2 and PD2 amended with 400 μM $ZnSO_4$ as previously described (De La Fuente et al., 2007) in five independent

experiments. Briefly, seven-day-old cultures were scraped from PW plates, suspended in 1ml of PD2, homogenized by pipetting, and introduced into the microfluidic chamber through tubing connected to syringes. After approximately 2 h at a medium flow speed of 0.25 µl min^{-1}, when the cells had attached, the flow speed was adjusted to 1.0 µl min^{-1} for 1 h to remove non-attached cells (remaining attached cells were considered to be in the lag growth phase). The medium flow rate was then sequentially increased every 1 min by 10 µl min^{-1} from 1 to ~ 200 µl min^{-1} to gradually remove attached cells. Medium flow was controlled by an automated syringe pump (Pico Plus; Harvard Apparatus, Holliston, MA). Image acquisition was done every five seconds with phase contrast optics as described above. The number of attached cells remaining in each frame was scored by using NIS-Elements software version 3.0 (Nikon, Melville, NY). Cell counts were averaged across the 12 frames collected each minute to obtain the count for each flow rate. The cell adhesion force was calculated using a computational model according to methods described previously by De La Fuente *et al.* (De La Fuente et al., 2007).

To determine the percentage of attached cells, *X. fastidiosa* was grown in 250 ml flasks containing 50 ml of PD2 or PD2 amended with 400 µM $ZnSO_4$ at 28°C and 150 rpm for 5 days. Planktonic and biofilm fractions were separated and biofilm was resuspended in 5 ml of phosphate-buffered saline (PBS). Two hundred microliter aliquots of the planktonic and biofilm phases were used for DNA extraction (Doyle J., 1987), and total cell numbers were quantified by qPCR as described above. The percentage of attached cells was calculated as follows: [# biofilm cells / (# of biofilm cells + # planktonic cells)*100]. Five replicates were used per treatment and two independent experiments were performed.

EPS quantification

EPS production was evaluated by two different methods: a modified gravimetric method (Barrere, 1986) and a phenol-sulfuric acid method (Dubois et al., 1951). For both methods, *X. fastidiosa* was grown in 250 ml flasks containing 50 ml of PD2 or PD2 amended with 400 µM $ZnSO_4$ and incubated at 28°C and 150 rpm for 5 days, a time when cells already entered the stationary growth phase. Planktonic and biofilm fractions were separated and the biofilm was resuspended in 5 ml PBS. EPS was extracted from both the planktonic and biofilm phases. A 20 ml aliquot of the planktonic phase was centrifuged at 11000x*g* for 30 minutes. Then, 10 ml of the supernatant were transferred to a new tube, and 125 µl of saturated KCl and 4 volumes of ethanol were added. Samples were incubated overnight at 4°C and centrifuged at 3200x*g* for 20 min. For the biofilm phase, a 2 ml aliquot of the biofilm suspension was centrifuged at 11000x*g* for 30 minutes. The supernatant was transferred to a new tube, and 25 µl of saturated KCl and 4 volumes of ethanol were added. Samples were incubated overnight at 4°C and centrifuged at 3200x*g* for 20 min.

For the gravimetric method, $O.D._{600nm}$ of planktonic and biofilm phases was recorded prior to the EPS extraction. The EPS precipitate was filtered through a 1.1 µm pore size glass microfiber filter (VWR, UK) and dried at 80°C to constant weight. The biofilm suspension (5 ml) was treated as described for planktonic cells. Due to the prevalence of cell clumps in the biofilm suspension after thorough homogenization, the measurements from planktonic and biofilm phases were considered not statistically comparable when using the gravimetric method. Three replicates were used per treatment and two independent experiments were performed.

For the phenol-sulfuric acid method, 200 µl aliquots of the planktonic and biofilm phases were used for DNA extraction and total cells were quantified by qPCR as described above. EPS precipitates were resuspended in water and total sugars were measured as previously described (Dubois et al., 1951). Five replicates were used per treatment and two independent experiments were performed.

Gene expression analysis of biofilm cells

The expression of various genes involved in polysaccharide and EPS synthesis (*gtaB*, *xanB*, *gumD*, and *gumH*) was analyzed by RT-qPCR (see Table 1 for primers and probes). Fifty milliliter conical tubes containing two glass slides and 10 ml of PD2 or PD2 amended with 400 µM $ZnSO_4$ were 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 by absorbance at 260 nm using a NanoDrop 2000 Spectrophotometer (Thermo Scientific, Waltham, MA), 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. One microliter of cDNA was used for qPCR with conditions as previously described. Fold change in gene expression was calculated by the $2^{-\Delta\Delta Ct}$ method (Livak, 2001). The 16S rDNA gene was used as the endogenous control to normalize gene expression (Chatterjee et al., 2010, Chatterjee et al., 2008c). Other assayed genes (*asnsS, gyrB, nuoA, dnaQ, merD, petC*)

were not suitable as endogenous controls of gene expression under our experimental conditions (data not shown). Three replicates were used for each treatment. For cells grown in PD2, biofilms from two tubes were pooled and considered as one replicate. For cells grown in Zn-amended PD2, biofilms from three tubes were pooled and considered one replicate. Two independent experiments were performed.

Table II-1. qPCR primers and probes used in this study.

Primer name	Sequence	Reference
HL5	AAGGCAATAAACGCGCACTA	(Francis et al., 2006)
HL6	GGTTTTGCTGACTGGCAACA	(Francis et al., 2006)
HLP	FAM-TGGCAGGCAGCAACGATACGGCT-BHQ	(Francis et al., 2006)
Xf16SF	CTCGCCACCCATGGTATTACTAC	(Choi et al., 2010)
Xf16SR	CTGGCGGCAGGCCTAAC	(Choi et al., 2010)
Xf16SP	FAM-ATGTGCTGCCGTCCGACTTGCATG-BHQ	(Choi et al., 2010)
gtaBF	TGCATCCACTCTCTCGTATTTC	This study
gtaBR	AAGGGCCGCATTACTCAAA	This study
gtaBP	FAM-ATATCTTGCCACTCAGGACATAGC-BHQ	This study
xanBF	TGATGCGCTTGACTTGGA	This study
xanBR	AGAAACCGATGACGCCTTATTAG	This study
xanBP	FAM-AACGATCTCTTTGACGGTCTGG-BHQ	This study
gumDF	GGCGTTGCATGTAATGCTGGATCA	This study
gumDR	GTGCTCAAATTTCGCTCGAT	This study
gumDP	FAM-AAACCCTCATGCCGCGACAGTGAAAT-BHQ	This study
gumHF	ATCAATCCAGCACTCATCGCCTCA	This study
gumHR	ATTGTAGTCCGTCACAGTCGCAGT	This study
gumHP	FAM-ATAACTGTTGTGCTGAGCCGCATGA-BHQ	This study

Assessment of biofilm structure by laser scanning confocal microscopy (LSCM)

X. fastidiosa strain KLN59.3 (Newman et al., 2003), which constitutively expresses green fluorescent protein, was inoculated in 50 ml conical tubes containing two glass slides and 10 ml of PD2 or PD2 amended with 400 μM $ZnSO_4$. Tubes were incubated at 28°C and 150 rpm for 13 days. Biofilm formed on the glass slides was stained with calcofluor white (MP Biomedicals, Illkirch, France) to stain polysaccharides as previously described (Clark et al., 2007). Calcofluor white stains beta-linked polysaccharides (Clark et al., 2007, Wood, 1980, Wood et al., 1983), and *X. fastidiosa* EPS has been shown to have both beta and alpha-linked polysaccharides (da Silva, 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.

Statistical analyses

Total, viable, and culturable cell counts were analyzed by ANOVA and means compared by Fisher's least significant difference (LSD) test. Data from the attachment ratio experiment were analyzed by t-test. Data from adhesion force experiments were evaluated by paired t-test. Data from EPS production measured by the gravimetric method were ln-transformed and analyzed by ANOVA and means compared by Fisher's LSD test, while data from the phenol-sulfuric acid method were transformed by ln (square root(x)) and analyzed likewise. All data transformations were performed to fit the data to the corresponding model assumptions. Fold changes in gene expression were analyzed by the t-test. Least-significant differences were

compared at a level of significance of $P \leq 0.05$. All analyses were conducted with SAS 9.2 (SAS Institute Inc. NC).

Results

Bacterial growth in test tubes.

The addition of Zn to the basal medium (PD2) in a range of 25 to 2000 μM had a negative effect on the growth of both planktonic cells and biofilm formed in batch cultures (Fig. II-1). Planktonic growth was significantly reduced as Zn was added up to 400 μM, where growth was reduced to approximately 15% of the control. At higher concentrations of added Zn, further reductions in growth were minimal. Biofilm showed a stepwise growth reduction with added Zn: it was reduced to approximately 60% of the control with 200 μM Zn and to approximately 10% of the control with 400 μM or higher Zn. Four-hundred micromolar added Zn was chosen for subsequent experiments because it represents the minimum concentration with a maximal effect on both planktonic and biofilm growth.

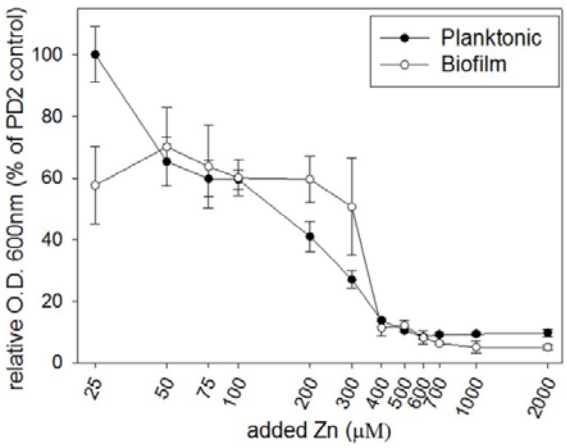

Figure II-1. Planktonic growth and biofilm formation in response to zinc supplementation.

X. fastidiosa was grown for 10 days in test tubes containing PD2 or PD2 amended with different concentrations of $ZnSO_4$ (shown in log scale). Growth of Xf cells in PD2 control medium (measured by $O.D._{600nm}$) was defined as 100% and growth in the Zn-amended media was expressed as a percentage relative to the PD2 control. Data shown is mean ± standard error (n=6). A combination of four independent experiments is shown.

Quantification of total, viable, and culturable cells over time.

X. fastidiosa grown in PD2 showed a decrease in the number of viable and culturable cells over time. For the planktonic phase, the maximum amount of viable and culturable cells was observed at three dpi, when total, viable and culturable cell numbers were non- significantly different (P >0.05). At five dpi, the number of viable and culturable cells were non-significantly different from each other but were lower than the total number of cells. At seven dpi and after, the total number of cells was significantly (P < 0.05) higher than the number of viable cells, which was higher than the number of culturable cells (Fig. II-2A). Biofilms grown in PD2 showed a similar tendency (Fig. II-2B). In the Zn-amended medium the viable

and culturable cell numbers were relatively stable throughout the time-course of the experiment. In contrast to what was observed in PD2, at three dpi the numbers of viable and culturable cells in the planktonic fraction were not different ($P < 0.05$) from each other, but were lower than the total number of cells ($P < 0.05$). From five dpi and after, the total number of cells was higher ($P < 0.05$) than the number of viable cells which in turn was significantly higher than the culturable cells (Fig. II-2C). Biofilms showed a similar trend (Fig. II-2D). In both phases (planktonic and biofilm cells), the significant differences between total and viable cell numbers (indicative of compromised cell membrane, thus likely cell death) were detected earlier in the Zn-amended PD2 (three dpi), compared to PD2 (five dpi). Likewise, the significant differences between viable cell numbers and culturable cells were detected earlier in PD2 amended with Zn (five dpi), compared to PD2 (seven dpi).

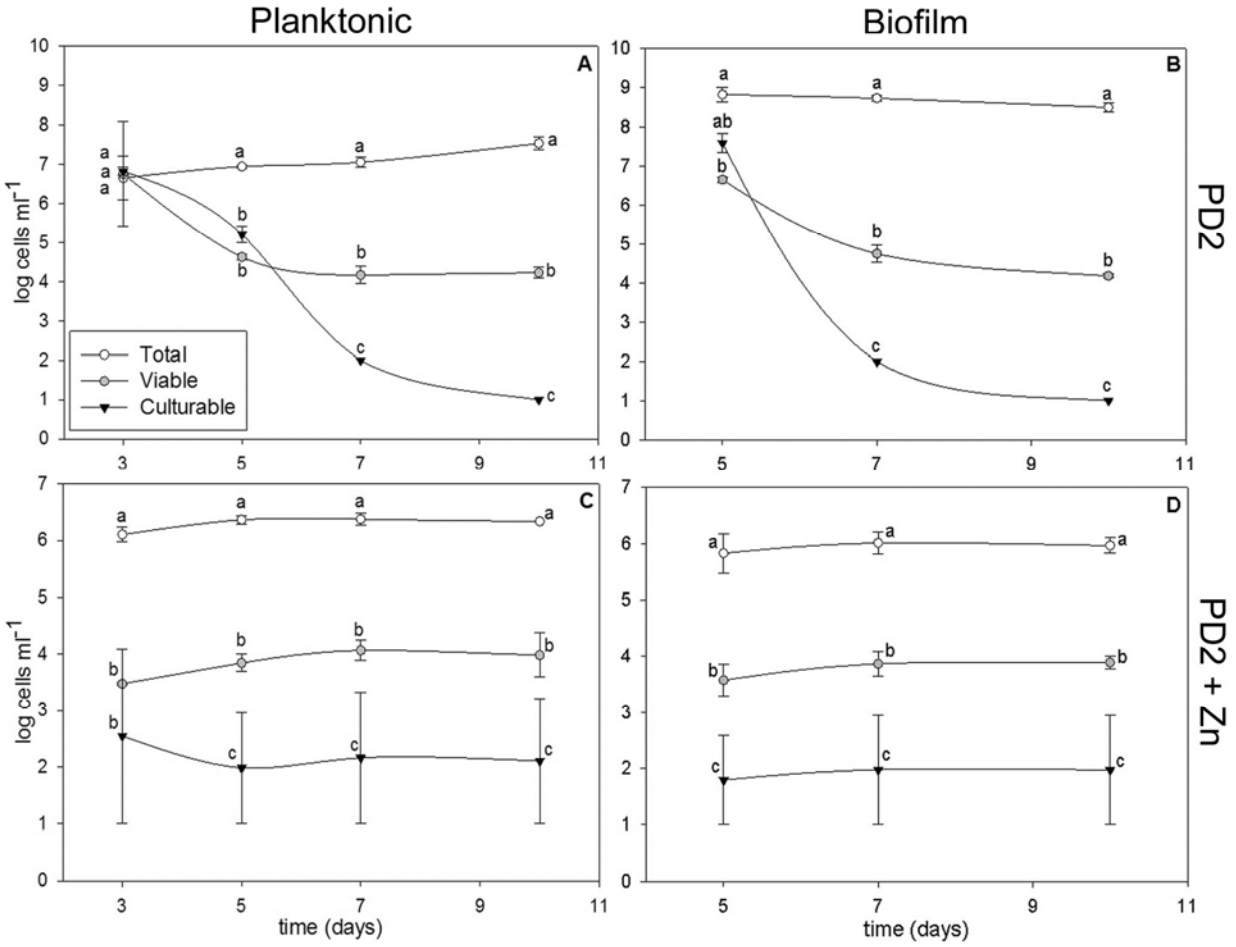

Figure II-2. Total, viable, and culturable cell counts.

X. fastidiosa was grown in flasks containing 50 ml of PD2 (A, B) or PD2 amended with 400µM of ZnSO4 (C, D) for 10 days. Xf cells were sampled at different time points and total cells (qPCR), viable cells (EMA-qPCR), and culturable cells (plated on PW) were quantified (see methods for details). Total, viable, and culturable cell numbers were measured at 3, 5, 7, and 10 dpi for the planktonic phase (A, C) or 5, 7 and 10 dpi for the biofilm phase (B, D). For each time point in the same graph, different letters indicate statistical difference ($P < 0.05$) according to ANOVA and least significant difference. Each data point in the figure represents mean ± SE (n=3) from one representative experiment.

Biofilm growth in microfluidic chambers.

When cells were incubated under flow conditions inside microfluidic chambers, cell aggregates were visible in the PD2 channel at five to seven dpi (Fig. II-3A), whereas in the Zn-amended channel the cell aggregates tended to be smaller or did not form at all (Fig. II-3B). When cells were re-injected overnight, the already formed aggregates in the PD2 channel grew only slightly (Figs. II-3C, II-3E), whereas in the Zn-amended channels dramatic growth of a biofilm-like structure was seen (Figs. II-3D, II-3F, Video S1). This biofilm-like structure covered most of the Zn-amended channel and was more resistant to shear force (i.e., it was harder to wash away by the media flow) than the aggregates formed in the PD2 channel (Video S2). Additionally, twitching was readily observed in the PD2 channel, whereas in most experiments it was not detected in the Zn-amended channel.

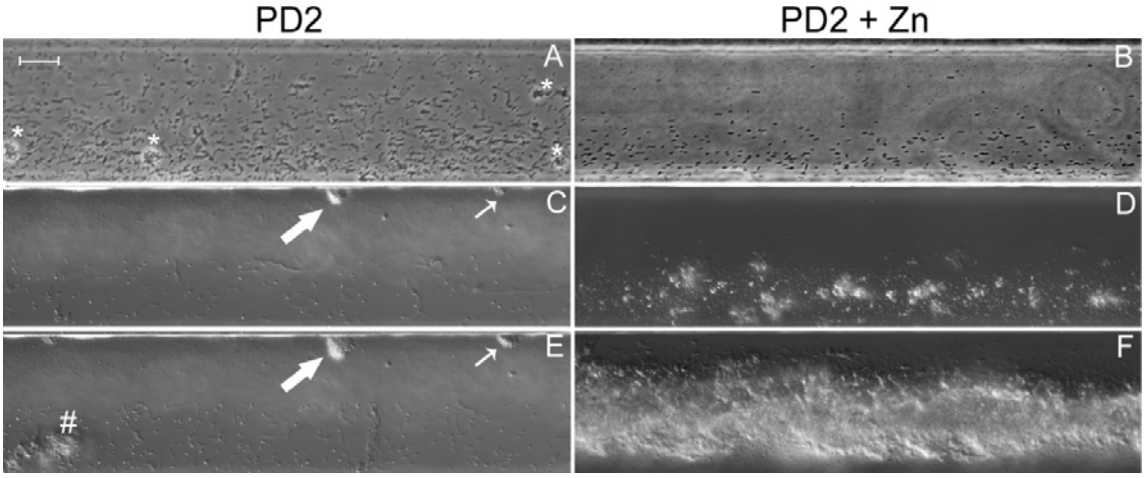

Figure II-3. Biofilm growth in microfluidic chambers.

Suspensions of *X. fastidiosa* ($O.D._{600nm}$= 0. 1) were injected overnight into microfluidic chambers containing PD2 (A, C, E) or PD2 amended with 400µM of $ZnSO_4$ (B, D, F) and incubated under a constant flow rate of 0.25 µl/min for 5-7 days (A, B). After cell aggregates (*) were formed in the PD2 channel, cells were re-injected in both the PD2 (C, E) and Zn-amended channels (D, F). Note that upon re-injection, aggregates formed in PD2 grow slightly (arrows in C and E) and new aggregates

appear (#). In contrast, massive aggregates were formed in the Zn-amended channels (D and E) upon re-inoculation. Pictures showed here were captured 2 hours (C, D) or 12h (E, F) after continuous re-injection of Xf cells. Scale bar shown in A represents 20 μm. A and B: phase-contrast optics, C-F: DIC optics.

Surface attachment.

Supplementation of PD2 with 400 μM $ZnSO_4$ tended to increase the adhesion force of *X. fastidiosa* cells to the glass inside microfluidic chambers, but this was not statistically significant ($P > 0.05$). The adhesion forces were 163±5 pN and 183±9 pN for PD2 and Zn-amended PD2 media, respectively (Fig. II-4A). Zn supplementation did have a significant effect on the percentage of cells that attached to the flask walls ($P < 0.05$). *X. fastidiosa* cells grown in PD2 had a much larger population in biofilms than in the planktonic phase, since 89± 3 % of the cells in the culture flask were attached to the walls (Fig. II-4B). The opposite trend was seen in the Zn-amended medium, where the planktonic population was higher than the biofilm population, and only 24±12 % of the cells were attached to the flask (Fig. II-4B).

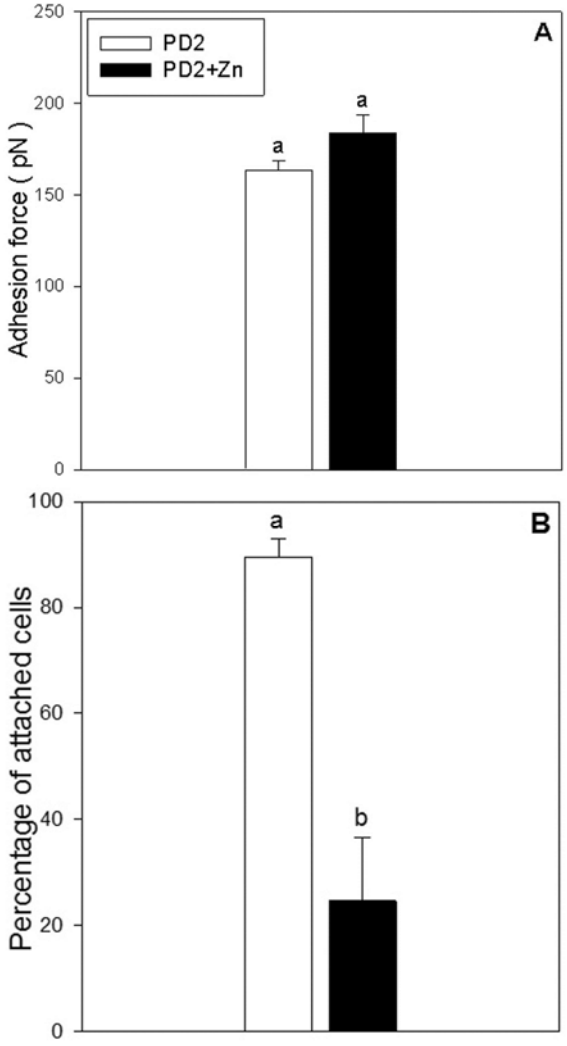

Figure II-4. Surface attachment in different concentration of Zn.

A) Adhesion force assessed in microfluidic chambers containing PD2 or PD2 amended with 400µM of $ZnSO_4$ (PD2+Zn). B) The percentage of attached cells [defined as: # biofilm cells / (# of biofilm cells + # planktonic cells)*100] was calculated for 50 ml batch cultures. Cells were grown for 5 days in PD2 or PD2+Zn. Number of planktonic and biofilm cells were quantified by qPCR. Data shown are mean ± standard error (n=5) from one representative experiment. The experiments were repeated five (A) and two (B) times independently. Different letters indicate statistical difference ($P < 0.05$) according to paired t-test.

Evaluation of EPS production.

EPS production in planktonic and biofilm phases as evaluated by the gravimetric and phenol-sulfuric acid methods was expressed as amount of EPS relative to the amount of bacterial cells. This standardization of EPS production was needed since bacterial growth is much higher in the PD2 control than in the Zn-amended medium (Fig. II-1). Results were expressed for the gravimetric method as g O.D. $_{600nm}^{-1}$ ml^{-1} and for the phenol-sulfuric acid method as µg glucose eq. cell^{-1}. For both methods, relative EPS production was always significantly higher (P <0.05) in the Zn-amended medium. In the biofilm phase, for both methods, relative production of EPS was two to three logs higher in the Zn-amended medium (Figs. II-5A, 2.5B). In the planktonic phase, the gravimetric method showed a two log increase in relative EPS production in the Zn amended medium whereas the phenol-sulfuric acid method showed less than one log increase (Figs. II-5A, II-5B). For cells grown in PD2, the phenol-sulfuric acid method (which allows planktonic vs. biofilm comparisons unlike the gravimetric method, see methods section for explanation) indicated that the relative EPS production in the biofilm is lower than in the planktonic phase. This may indicate that a large proportion of the EPS produced in the biofilm sheds away and is dissolved in the growth medium.

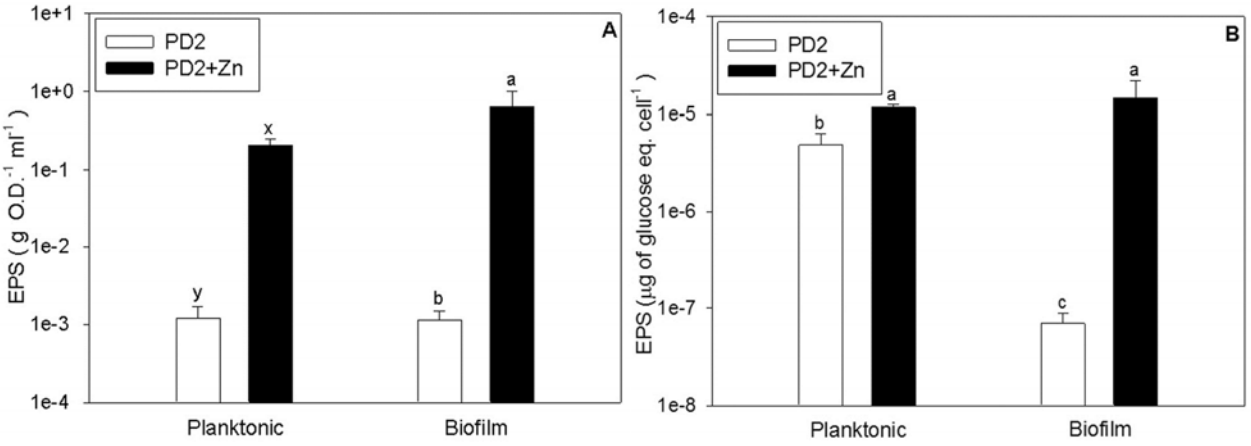

Figure II-5. EPS quantification.

EPS quantification of *X. fastidiosa* cells grown for 5 days in flasks containing 50 ml of PD2 or PD2 amended with 400µM of $ZnSO_4$ (PD2+Zn). A gravimetric method (A) and a phenol-sulfuric acid method (B) were used to evaluate EPS production in planktonic and biofilm phases. Results are expressed as EPS production relative to cell growth. Data shown are mean ± standard error (A: n=3; B: n=5) from one representative experiment. For each graph, different letters indicate statistical differences ($P < 0.05$) according to ANOVA and least significant difference. In graph A, planktonic cells were analyzed separately from biofilm cells as noted in materials and methods.

Expression of genes involved in EPS biosynthesis.

The expression of *gtaB, xanB, gumD*, and *gumH*, was evaluated in PD2 and PD2 amended with Zn. Zinc amendment produced a significant increase in the expression of *gtaB* and *xanB* (fold change: 2.6± 0.5 and 5.7±07, respectively). Expression of *gumD* and *gumH* was not altered by amendment with Zn (fold change: 1.2± 0 and 0.8±0.2, respectively).

Biofilm structure assessed by LSCM.

Examination of *X. fastidiosa* biofilms by confocal microscopy allowed for observation of their three-dimensional structure. Biofilms grown in PD2 formed a heterogeneous (though fairly compact) structure, with cells being oriented horizontally and relatively few scattered cells observed. EPS (stained bluish-white by calcofluor white) was observed in patches, intermingled with some cell groups. In some cases, EPS formed big clumps that surrounded cell masses (Fig. II-6A). In the Zn-amended medium, biofilms consisted of more scattered, horizontally-oriented cells, and compact groups of cells were scarce. EPS co-localized with the cells, and no clumps of EPS were observed surrounding cell aggregates (Fig. II-6B).

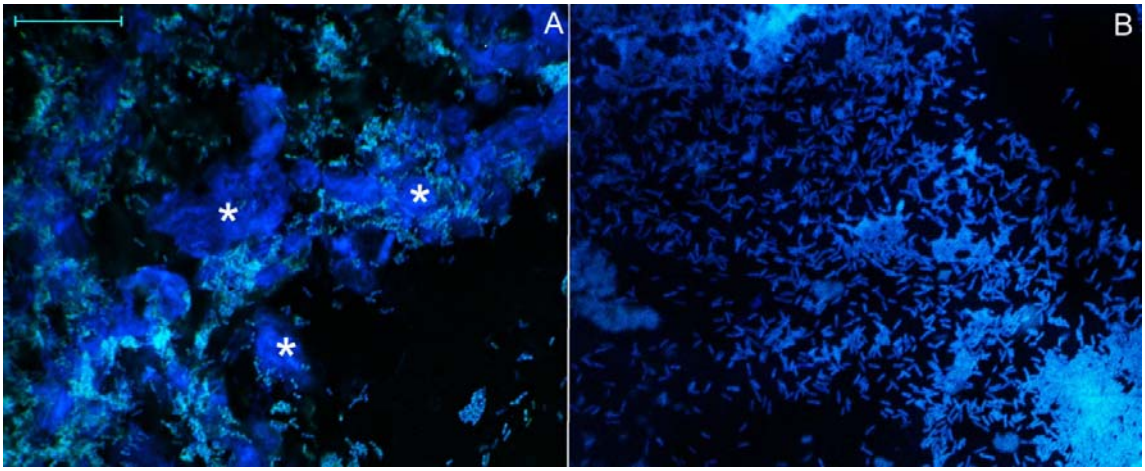

Figure II-6. Structure of *X. fastidiosa* biofilms grown in different Zn concentrations.

Biofilms were formed on glass slides immersed in batch cultures of an Xf strain expressing green fluorescent protein (blue-green color in figures) grown in (A) PD2 or (B) PD2 amended with 400µM of $ZnSO_4$. 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. Note the abundant polysaccharide masses between cells in A (*). Scale bar shown in A corresponds to 10 µm for both images.

Discussion

In the present study it was established that Zn has pleiotropic effects on *X. fastidiosa*, affecting growth, biofilm, and survival strategies *in vitro*. Under flow conditions and with constant bacterial feeding into the system, Zn triggers the formation of very compact biofilm structures that are more resistant to shear force than the structures formed in non-amended medium. In this regard, the use of microfluidic chambers played a pivotal role, suggesting the augmentation of *X. fastidiosa* adhesive properties in response to Zn supplementation that was correlated with increased EPS formation. Although this metal induces a large increase in the production of EPS, most of the bacterial cells remain in the planktonic fraction in batch cultures. These results suggest that Zn promotes cell-to-cell attachment (shown previously by our group) (Cobine et al., 2013) while it decreases surface-cell attachment. The former may be an important factor in the Zn-induced biofilm structures observed in microfluidic chambers after re-inoculation.

Zn is essential for all living organisms as it is required for stabilization of protein and membrane structure (Andreini et al., 2006, Chvapil, 1973). However, at high concentrations it can be toxic due to inhibition of the electron transport chain or the metabolism of other metals (Chvapil, 1973, Blencowe & Morby, 2003, McDevitt et al., 2011). Studies in various bacteria have shown that Zn can also influence virulence factors (Fones & Preston, 2013, De La Fuente *et al. in press*). In *Escherichia coli* the two-component regulator BasS/*R* that senses Fe and Zn, promotes the expression of genes involved in lipopolysacharide (LPS) modification, biofilm formation and stress responses (Ogasawara et al., 2012). In *Xanthomonas* spp., mutations in the zinc uptake regulator *zur* impaired production of EPS and virulence (Tang et al., 2005, Yang et al., 2007). To the best of our knowledge, this is the first study in which it

was demonstrated that Zn alters expression of genes involved in biosynthesis of EPS, augments EPS production, and affects culturability in a bacterium. The observed inhibition of growth caused by higher concentrations of Zn in the media is in agreement with previous reports (Cobine et al., 2013, Cruz et al., 2012, Brady, 2010, Malavolta, 2005). Zn tolerance/resistance is particularly significant for the *X. fastidiosa* life cycle since Zn-containing fungicides (Datnoff et al., 2007, Barker & Pilbeam, 2007, Wilcox, 2013, Gubler et al., 2009), and foliar Zn sprays are commonly used for fertilization (Datnoff et al., 2007, Marschner, 1995, Barker & Pilbeam, 2007), therefore increasing Zn content in the host.

Increased EPS production was detected when Zn was added to PD2 medium. However, no EPS clumps were seen in the biofilm in batch cultures, in which EPS only covered the cells. This could be attributed to greater shedding of EPS from cells grown under high Zn concentrations. The observation that a large proportion of the EPS produced by *X. fastidiosa* can be loosely associated with the cells has been made previously (Roper et al., 2007a, Killiny & Almeida, 2009). This phenomenon could also explain the observation that when cells were grown in PD2, higher relative EPS production (glucose eq. $cell^{-1}$) was detected in the planktonic phase, since EPS shedding from the biofilm fractions could end up in solution with the planktonic cells. In general, both methods employed for EPS quantification gave similar results. The differences in magnitude of the increase in the planktonic phase seen between methods could be due to co-precipitation of compounds others than polysaccharides during the extraction process that could affect the weight of the precipitate. The phenol-sulfuric acid method, however, is less likely to be affected by non-specific compounds. The increased EPS production in PD2 + Zn is likely responsible for the production of the massive aggregates formed upon re-inoculation of stationary phase *X. fastidiosa* cells into the microfluidic chambers. When cells were in the lag growth phase, Zn did not affect adhesion force to

surfaces, indicating that accumulation of metabolic products is required for this effect. All these results together indicate that the resilient biofilms observed in microfluidic chambers using PD2+Zn medium are due to stronger cell-to-cell attachment explained by higher EPS production.

Bacterial EPS synthesis can be divided into three stages: i) conversion of monosaccharides to nucleotidyl derivative precursors, ii) assembly of the oligosaccharide repetitive unit attached to a polyprenol phosphate carrier, and iii) polymerization of the repetitive unit and secretion of the polymer (da Silva, 2001). Here, expression of genes involved in EPS synthesis in *X. fastidiosa* (*gtaB*, *xanB*, *gumD*, *gumH*) was analyzed to identify changes in expression induced by the presence of Zn. GtaB (UTP-glucose-1-phosphate uridylyltransferase) transfers a UDP unit to glucose-1-P. XanB (phosphomannose isomerase-GDP-mannose pyrophosphorylase) transfers a GDP unit to mannose-1-P. GumD (UMP-glucosyltransferase) and GumH (GDP-mannosyltransferase) add the first glucose and mannose units, respectively, to the EPS repeating unit (Killiny et al., 2013, da Silva, 2001). As shown by RT-qPCR, Zn affects the initial steps of EPS synthesis in *X. fastidiosa*, increasing the availability of nucleotidyl precursors. *gtaB* and *xanB* are involved in EPS synthesis as well as other biosynthetic pathways. UDP-glucose, the product of GtaB is required for xanthan gum production in *X. campestris* and for the synthesis of the capsular exopolysaccharide in *Streptococcus pneumoniae* as well as for the synthesis of LPS and periplasmic cyclic glucans in many bacterial species (Wei et al., 1996, Mollerach et al., 1998, Sundararajan et al., 1962, Bohin, 2000, Breedveld & Miller, 1994). *xanB* is essential for EPS, LPS, and flagellar-mediated motility in *X. campestris* (Hotte et al., 1990, Koplin et al., 1992), and for twitching motility in *Stenotrophomonas maltophilia* (Huang et al., 2006). All of the biosynthetic pathways mentioned above, in which *gtaB* and *xanB* participate, are known to affect membrane

structure and function of its associated apparatuses such as type IV pilli, as well as cell adhesive proprieties (Whitchurch et al., 1996, Huang et al., 2006, Raetz & Whitfield, 2002, Clifford et al., 2013, Bohin, 2000, Breedveld & Miller, 1994, Denny, 1995). Therefore, the increased expression of *gtaB* and *xanB* induced by Zn could disturb outer membrane properties of *X. fastidiosa*, affecting binding of EPS to the cells surface and also account for the observed reduction in attachment to glass surfaces and lack of twitching motility and auto-aggregation inside microfluidic chambers (before re-inoculation). Both twitching motility and auto-aggregation are dependent on type IV pili (Meng et al., 2005, De La Fuente et al., 2008). Interestingly, the XanB protein of several bacteria has a Zn-binding motif (Sousa et al., 2008), which is conserved in *X. fastidiosa* (data not shown). This may explain the *xanB* up-regulation observed here in Zn-amended PD2.

Modification of the outer membrane and increased EPS production are likely resistance mechanisms against elevated Zn concentrations. LPS is known to bind cations and protect against antibacterial compounds and other environmental stressors (Vaara, 1992, Raetz & Whitfield, 2002, Clifford et al., 2013). The same is truth for EPSs, which binding cations to their negatively-charged groups (Geddie, 1993, Wolfaardt, 1999). The negatively charged residues in the EPS from *X. fastidiosa* (Killiny et al., 2013, da Silva, 2001) could chelate Zn, that was shown to accumulate Zn in biofilms (Cobine et al., 2013), thus preventing accumulation of toxic levels inside the bacterial cell. A similar well-characterized case involves the response of *E. amylovora* to copper, where this bacterium increases EPS production upon contact with high levels of Cu (Bereswill, 1998). Its EPS is able to chelate Cu, delays the entrance into the Cu-induced viable but non-culturable state (VBNC), and can be used as a carbon source by cells in this state (Ordax et al., 2010). *X. fastidiosa* may respond in a similar manner to high Zn levels.

The VBNC state has been defined as a state in which bacteria fail to grow and develop into colonies on media on which they would normally grow, although they remain alive (Oliver, 2000). Most investigators hypothesize that the VBNC state is a survival strategy against stressful conditions including starvation, improper growth temperatures, and/or heavy metals (Oliver, 2010). This phenomenon seems to be widespread in bacteria, including aquatic bacteria, animal pathogens, and plant pathogens (Oliver, 2010, Oliver, 1995, Alexander et al., 1999, Grey & Steck, 2001a, Grey & Steck, 2001b, Wilson, 2000). It was shown here that when *X. fastidiosa* is grown in batch cultures, its ability to be cultured on a conventional solid medium is quickly reduced, though a large proportion of the total population remains viable according to EMA-qPCR. When Zn was added to PD2, an early decrease in the viability and culturability of *X. fastidiosa* was seen. These results suggest that *X. fastidiosa* may be able to enter a VBNC state *in vitro* and are consistent with observations from other bacteria when incubated in the presence of compounds that induce the VBNC such as Cu (Alexander et al., 1999, del Campo et al., 2009, Ordax et al., 2006). Interestingly, *Enterococcus* spp. do not form biofilms after entering the VBNC state but continue producing EPS (Lleo et al., 2007), a similar response to what was observed when *X. fastidiosa* was exposed to high Zn concentrations. To the best of our knowledge, there are no reports on Zn inducing the VBNC state in bacteria, but *Vibrio cholerae* is able to tolerate higher levels of Zn when in the VBNC state than when in the exponential growth phase (Chaiyanan et al., 2001). Since the cell viability method employed here (EMA-qPCR) is based on membrane integrity measurements, as is BacLight™, another common method (Rudi, 2005, Nocker & Camper, 2006, Boulos et al., 1999, Mesa et al., 2003), the possibility that non-viable cells retain membrane integrity for a considerable time after death cannot be ruled out (Nocker & Camper, 2006). Evaluations of metabolic activity of *X. fastidiosa* under various culture conditions may elucidate whether *X. fastidiosa* is truly capable of entering the VBNC state. Although EMA treatment is an

imperfect measure of viability (Nocker & Camper, 2006, Flekna et al., 2007), the lack of significant differences between viable and culturable cells at earlier time points and the fact that loss of culturability is associated with loss of viability indicate that the observed differences at later time points between viable and culturable cells are unlikely due to the limitations of the method. The VBNC state is relevant for virulence since cells in this state may remain virulent or "resuscitate" and reacquire virulence once harsh conditions disappear, or upon contact with the host (del Campo et al., 2009, Ordax et al., 2006, Grey & Steck, 2001b).

Collectively, results from this study may explain the inconsistencies in disease severity observed when Zn drenches were applied to *X. fastidiosa*-infected grapevines (Brady, 2010). Elevated Zn concentrations could prevent large *X. fastidiosa* populations from developing; while at the same time aggregates and EPS could accumulate that aggravate symptoms. If the Zn-induced aggregates are formed in planta, they would likely increase insect transmissibility, since biofilm formation is required for this process (Newman et al., 2004). The construction of mutants altered in Zn homeostasis will certainly be important to identify both the effects of this metal during infection and the signaling networks in which it is involved. Together with other studies (Zaini et al., 2008, Cruz et al., 2012, Cobine et al., 2013, De La Fuente et al., 2013), this study highlights the importance that mineral elements have for the regulation of virulence traits in vascular pathogens. Ultimately, careful selection of planting site, rootstock, and fertilization regime could be used to adjust/modify the host mineral composition for disease management.

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Chapter III
Zinc homeostasis is required for regulation of virulence in *Xylella fastidiosa*

Abstract

Xylella fastidiosa is a bacterial plant pathogen that exclusively colonizes the xylem vessels of its plants hosts and the foregut of its insect vectors. Effects on diseased plants include chlorosis, leaf scorching, water stress, and altered mineral composition of the leaves (ionome). Inside xylems vessels, the bacterium grows systemically, forming biofilms. Biofilms are structured communities of bacteria attached to a surface and surrounded by a secreted matrix constituted mainly of exopolysaccharides (EPS). In *X. fastidiosa*, the production of EPS is essential for pathogenicity, and biofilm and EPS production are greatly influenced by the mineral elements in the growth medium. Particularly, zinc decreases survival and biofilm production, while it increases the production of EPS in this bacterium. Here, the effects of this metal on disease development were evaluated by knocking out two genes involved in Zn homeostasis, *zur* and *czcD*. Both mutant strains were affected in their abilities to grow in media supplemented with Zn and in xylem sap compared to the wild type (WT). In media with low or no Zn supplementation, the *zur* mutant exhibited increased biofilm production, whereas biofilm was diminished at high levels of Zn supplementation. The *czcD* mutant showed decreased biofilm production only at high levels of Zn supplementation. Both mutant strains accumulated higher intracellular levels of Zn than the WT, and EPS production was reduced in Zn-supplemented media. Plants inoculated with both mutants had reduced bacterial populations and caused reduced symptoms compared to plants inoculated with the WT. Additionally, both mutants failed to alter the leaf ionome of inoculated plants, as was seen plants inoculated with WT bacterium. These results demonstrate that zinc detoxification is necessary for and full virulence in this bacterium.

Introduction

The bacterium *Xylella fastidiosa* is a member of the Xanthomonadaceae (γ-Proteobacteria) that colonizes only the xylem of its host plants and the foregut of xylem-feeding sharpshooter insect vectors (Cicadellidae) (Hopkins & Purcell, 2002, Chatterjee et al., 2008). *X. fastidiosa* is associated with a wide range of plant diseases causing great economic losses, including Pierce's disease of grapevine (PD), citrus variegated chlorosis (CVC), almond leaf scorch (Hopkins & Purcell, 2002), and bacterial leaf scorch of blueberry (Chang et al., 2009).

Symptoms of *X. fastidiosa* infection vary depending on the host and include leaf chlorosis, marginal scorching, and/or dwarfing, and are associated with systemic blockage of xylem vessels (Hopkins & Purcell, 2002). Symptoms have been attributed to prolonged water stress caused by *X. fastidiosa* growth and biofilm formation in the xylem vessels (Goodwin et al., 1988). Biofilms are structured, surface-associated communities of bacteria surrounded by an extracellular matrix that protects bacteria from harsh environmental conditions (Costerton et al., 1995, Branda et al., 2005) and, in the case of *X. fastidiosa*, are also important for adhesion to xylem vessels and insect transmission (Chatterjee et al., 2008). The biofilm matrix can contain exopolysaccharides (EPS), proteins, and/or nucleic acids (Branda et al., 2005). EPS protects bacteria from contact with toxic molecules and can induce symptoms such as wilt in plant hosts, as in *Ralstonia solanacearum* or *Erwinia amylovora* infections (Denny, 1995). In *X. fastidiosa*, it was recently demonstrated that EPS production is fundamental for biofilm production, pathogenicity, and insect transmission (Killiny et al., 2013) .

Plants infected with *X. fastidiosa* experience water stress as well as altered expression of genes involved in nutrient uptake (Choi H. K., 2013). Likewise, analysis of the mineral composition of diseased plants shows increased levels of calcium and reduced levels of potassium and phosphorus (Goodwin et al., 1988, Andersen & French, 1987, Silva-Stenico et al., 2009, De La Fuente et al., 2013). This alteration of the mineral content of the host is important for *X. fastidiosa,* since mineral composition of the growth medium greatly affects the growth characteristics of this bacterium. For instance, amendment of synthetic media with xylem sap promotes surface attachment and production of biofilms with compact architecture (Zaini et al., 2009). In addition, supplementation of synthetic media with different metals alters growth and virulence traits of *X. fastidiosa.* Supplementation with calcium promotes attachment to surfaces, cell to cell aggregation, biofilm formation, and twitching motility (Cruz et al., 2012). Supplementation with iron promotes expression of genes involved in the synthesis of type I and type IV pili and the production of bacteriocins (Zaini et al., 2008). Supplementation with copper promotes cell aggregation at low concentrations, but it inhibits growth at high concentrations (Cobine et al., 2013).

In Chapter II of this thesis, it was demonstrated that supplementation of synthetic culture media with zinc reduces growth and induces loss of viability and culturability of *X. fastidiosa*, whereas it increases EPS production. However, there are contradictory reports in regards to the effect of this metal on disease progression. Most preliminary reports published in non-peer reviewed journals, have established a negative correlation between zinc levels in the host plant and disease severity. Growth of citrus trees in a hydroponic system without supply of Zn increased the bacterial populations compared to plants grown in media with normal supply of Zn (Malavolta, 2005). Application of Zn drenches to grapevines was shown to decrease bacterial populations; however, the effects on disease development were inconsistent (Brady,

2010). On the contrary, Zn concentrations in grapevine xylem sap have also been found to be positively correlated with growth of *X. fastidiosa* (Andersen et al., 2007).

In *Xanthomonas* spp., bacteria closely related to *X. fastidiosa*, correct Zn homeostasis is required for proper host colonization and full virulence. In *X. campestris* pv. *campestris* and *X. oryzae* pv. *oryzae*, the zinc uptake regulator gene, *zur*, is required for growth in media supplemented with zinc and for production of EPS. Accordingly, these *zur* mutants have reduced virulence (Tang et al., 2005, Yang et al., 2007). In addition, *zur* controls the expression of *czcD*, a member of the Cation Diffusion Facilitator (CDF) family that is required for Zn efflux (Nies, 2003, Huang & Tang, 2008). Growth of *X. campestris* pv. *campestris czcD* mutant is inhibited in media supplemented with zinc, at the same concentration that inhibit the growth of *X. campestris* pv. *campestris zur* mutant (Huang & Tang, 2008). Taken together, these data suggest that *zur* exerts its effect on the detoxification of toxic Zn levels, at least in part, by enhancing the expression of *czcD* (Huang & Tang, 2008). Unfortunately, the virulence of *X. campestris* pv. *campestris czcD* mutant has not been evaluated.

The objective of the present study was to construct *X. fastidiosa* mutants deficient in the homeostasis of zinc, by creating *zur* and *czcD* gene knockouts, and evaluate the effects of these mutations on disease development in planta. Bacterial growth and biofilm production was evaluated in media with different concentrations of zinc, including grapevine xylem sap. EPS production was evaluated in synthetic media with different zinc concentrations and virulence was evaluated in a model host, *Nicotiana tabacum* (tobacco). Results show that *zur* and *czcD* are necessary for growth of *X. fastidiosa* in media supplemented with Zn and in xylem sap. These genes are also required for normal EPS production and colonization of the

host plant. Plants inoculated with *zur* and *czcD* mutants also exhibit reduced symptoms and reduced alteration of the leaf mineral composition compared to plants inoculated with the *X. fastidiosa* wild-type (WT), demonstrating that zinc detoxification is necessary for full virulence in this bacterium.

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. Bacterial stocks were stored in PD2 (Davis et al., 1981a) broth with 20% glycerol at -80°C. All strains were grown on PW agar (Davis et al., 1980) with or without kanamycin (30 μg/ml).

Construction of mutant strains

Homologous recombination was used to generate *X. fastidiosa zur* (NC_004556) and *czcD* (NC_004556) null mutants, and fusion PCR was used to construct the knockout vectors. First, *X. fastidiosa* WT genomic DNA was extracted using a modified cetyltrimethylammonium bromide (CTAB) protocol (Doyle & Doyle, 1987). For the construction of the *zur* knockout vector, polymerase chain reaction (PCR) was performed to amplify ~7500 bp upstream and downstream of the *zur* coding region. All PCR reactions were conducted using the Phusion High-Fidelity PCR Kit (New England Biolabs, Ipswich, MA) following manufacturer's instructions. All primers (Table III-1) were designed using Primer3 (Rozen S, 1998).The upstream and downstream fragments flanking the *zur* coding region were amplified using primers ZUPF/ZUPR (upstream) or ZDOF/ZDOR (downstream). ZUPR and ZDOF contain AscI restriction sites, which are needed for subsequent insertion of a kanamycin cassette (see

below). PCR was performed using the following parameters: 98°C for 10 sec, followed by 35 cycles of 98°C for 10 sec, 65°C for 30 sec, and 72°C for 1 min, and a final extension step of 72°C for 5 min. The amplified segments were visualized by gel electrophoresis. PCR products were purified using the PCRExtract Mini Kit (5 PRIME, Gaithersburg, MD) according to manufacturer's instructions. Subsequently, 1 ng of each of the previous purified PCR products were used as templates for fusion PCR using primers ZIF/ZIR. PCR was performed using the following parameters: 98°C for 10 sec, followed by 35 cycles of 98°C for 10 sec, 65°C for 30 sec and 72°C for 2 min, and a final extension step of 72°C for 5 min. The amplified segments were visualized by gel electrophoresis and the fusion product (1.2 Kbp) was gel purified using the Agarose GelExtract Mini Kit (5 PRIME, Gaithersburg, MD) according to the manufacturer's instructions. The purified product was then cloned into a pJET1.2/blunt cloning vector (Thermo Fisher Scientific, Pittsburgh, PA) following manufacturer's instructions. The resulting plasmid, pJET_*zur*, was transformed into *Escherichia coli* NEB 5-alpha (New England BioLabs, Ipswich, MA) and positive clones were selected in Luria-Bertani (LB) broth with ampicillin (100 µg/ml). A kanamycin cassette was amplified from pUC4K, with primers containing the AscI restriction site sequence, and cloned into pJET1.2 to produce pJET_kan and transformed into *Escherichia coli* NEB 5-alpha (New England BioLabs, Ipswich, MA). Positive clones were selected in LB broth with kanamicyn (50 µg/ml). pJET_*zur* and pJET_kan were digested with AscI. pJET_*zur* was dephosphorylated with shrimp alkaline phosphatase (Affymetrix, USB, Santa Clara, CA) for 1 hour at 37°C followed by 15 min at 65°C. pJET_*zur* and the kanamycin cassette released from the pJET_kan AscI digestion were then ligated with T4 ligase to create pJET_*zur*_kan (3h at RT).

The czcD knock out vector, pJET_*czcD*_kan, was constructed as described above using the primers CUPF/CUPR and CDOF/CDOR for amplification of the upstream and downstream

segments of the *czcD* coding region respectively. Fusion PCR was conducted using the primers CIF/CIR.

pJET_*zur*_kan and pJET_*czcD*_kan were electroporated into *E. coli* EAM1, which expresses a *X. fastidiosa* methyltransferase (PD_1607) (Matsumoto & Igo, 2010), in a 2 mm cuvette (Eppendorf, Hauppauge, NY) at 2.5 kV for 5.7 ms. Positive clones were selected from LB agar plates containing ampicillin (100 µg/ml) and kanamycin (50 µg/ml) incubated overnight at 37°C. For methylation of pJET_*zur*_kan and pJET_*czcD*_kan plasmids, *E. coli* EAM1 cells containing the corresponding plasmids were grown at 37°C and 200 rpm overnight in LB containing IPTG (1mM), ampicillin (100 µg/ml), and kanamycin (50µg/ml); plasmid DNA was extracted using the GeneJET Plasmid Miniprep Kit (Thermo Scientific, Pittsburg, PA).

Electrocompetent *X. fastidiosa* Temecula (WT) cells were prepared as previously described (Matsumoto et al., 2009). Fifty microliters of an electrocompetent *X. fastidiosa* suspension and 200 ng of methylated plasmid DNA were transferred to a 2 mm cuvette in ice (Eppendorf, Hauppauge, NY) and electroporated at 2.5 kV for 5.7 ms. Immediately, 1 ml of PD3 medium (Davis et al., 1981b) was added to the transformed cells, which were incubated at 28°C and 150 rpm overnight. Two hundred microliters of the culture were then spread-plated on PD3G solid media (Davis et al., 1981a) (solidified with Gelrite instead of agar) containing 30 µg ml^{-1} of kanamycin. Plates were incubated at 28°C until transformants were visible (~10-15 days). To confirm the mutations, DNA was extracted from resulting transformants using the previously mentioned CTAB protocol, PCR-amplified with primers designed to amplify the regions flanking recombination region (ZchUPF, ZchDOWNR or CchUPF, CchDOWNR) paired with primers designed from the kanamycin cassette (ZchUPR, ZchDOWNF or CchUPR, CchDOWNF) (Table III-1), and sequenced (Eurofins MWG Operon, Huntsville,

AL). Sequences were BLASTed against the *X. fastidiosa* Temecula genome (GenBank) to confirm the correct insertion of the kanamycin cassette and the absence of any sequence mismatches. Additionally, primers were designed to amplify an internal 400bp region of *zur* (ZkoF/ZkoR) or *czcD* (CkoF/CkoR) coding regions. In this screening, gene deletions yielded negative PCR amplifications.

Table III-1. PCR primers used for mutagenesis.

Primer	function/target	Sequence 5'-3' *
Mutagenesis		
ZUPF	Upstream *zur*	GACCTGCAGACGGCAAATCAACCTC
ZUPR	Upstream *zur*	CTTTGGGAGCGGATGGGCGCGCCTTCCGTGGGATTCA
ZDOF	Downstream *zur*	TGAATCCCACGGAAGGCGCGCCCATCCGCTCCCAAAG
ZDOR	Downstream *zur*	AATCTGCAGTCACTGGGACCACCTC
ZIF	Fusion	TCACTGCAGACGGCTGGGCTGGTC
ZIR	Fusion	AAACTGCAGCACTGCGGCGTTGCT
CUPF	Upstream *czcD*	ATACTGCAGCGGACGCACTGAAGG
CUPR	Upstream *czcD*	GTTGTGGGCTATTGGCGCGCCACGACACCCCTGCTT
CDOF	Downstream *czcD*	AAGCAGGGGTGTCGTGGCGCGCCAATAGCCCACAAC
CDOR	Downstream *czcD*	GTTCTGCAGCGATGCCTTCTTGACG
CIF	Fusion	AATCTGCAGAAATGGGTGCCGAAG
CIR	Fusion	TAACTGCAGGCTGGATGCGCGT
KanAscIF	Kan resistance cassette	TTAGGCGCGCCGTCTGCCTCGTGAAG
KanAscIR	Kan resistance cassette	AAAGGCGCGCCAAGCCACGTTGTGT
Confirmation of mutations		
ZkoF	*zur* deletion	GTGGACTACGCCTGACACCT
ZkoR	*zur* deletion	CGGTTGGGTCGTGTTGTG
ZchUPF	Upstream *zur* deletion	CGCATCTACCGATGATCCTG
ZchUPR	Upstream *zur* deletion	GCGAGCCCATTTATACCCAT
ZchDOF	Downstream *zur* deletion	CTGGTCCACCTACAACAAAGC
ZchDOR	Downstream *zur* deletion	AAGTCATCACCTCGCATAAC
CkoF	*czcD* deletion	CGAATCCACCCAATACCAAC
CkoR	*cacD* deletion	GAGTGTCCTAGTGGCCGAAA
CchUPF	Upstream *czcD* deletion	CCAAAGCGAAAGGACTCAAC
CchUPR	Upstream *czcD* deletion	TCCCGTTGAATATGGCTCA
CchDOF	Downstream *czcD* deletion	CGTGGCTCCCTCACTTTCT
CchDOR	Downstream *czcD* deletion	GTGTTGCAGCGTCCTTTAG

* AscI restriction site is underlined

Grapevine xylem sap collection

Xylem sap from grapevines (*Vitis* vinifera cv. 'Chardonnay') was collected by bleeding pruned vines in the spring of 2012 from a vineyard in Dahlonega, Georgia. Collected sap was filter-sterilized (0.2 μm) and stored at -80°C.

Bacterial growth in media with different zinc concentrations

Growth curves were obtained for *X. fastidiosa* WT, *zur*, and *czcD* grown in 96-well plates containing 200 μl of different media: PD2, PD2 + Zn (amended with 25, 100, and 400 μM $ZnSO_4$), 50% SAP (equal parts PD2 and xylem sap), and SAP (pure xylem sap). Seven-day-old *X. fastidiosa* cells collected from PW or PW + kanamycin solid media were used to inoculate media to an initial $O.D._{600nm}$ of 0.01. Plates were incubated at 28°C and 150 rpm for 7 days. $O.D._{600nm}$ was recorded daily for each strain–medium combination, with six replicates. On day seven, biofilm growth was evaluated by the crystal violet assay as described previously (Zaini et al., 2009). Briefly, after planktonic growth was recorded, the remaining broth was removed and the plates were gently rinsed with distilled water twice. Biofilms were stained with 240 μl of 0.1% crystal violet for 20 min. Plates were rinsed with distilled water as above, 240 μl of 6:4 (vol/vol) acetone/ethanol were added, and the plates were agitated at 150 rpm for 5 min. The $O.D._{600\ nm}$ of the resulting acetone/ethanol solution was the recorded. This experiment was performed once.

Bacterial zinc accumulation

For measurement of *X. fastidiosa* intracellular Zn content, seven-day-old *X. fastidiosa* WT, *zur*, and *czcD* cells were collected from PW or PW + kanamycin solid media and used to

inoculate 250 ml flasks containing 50 ml of PD2 to an initial $O.D._{600nm}$ of 0.01, with five replicate flasks per strain. Flasks were incubated at 28°C and 150 rpm for 3 days. The $ZnSO_4$ concentration of the broth was adjusted to 100 μM and the flasks were further incubated overnight. Planktonic cells were harvested by centrifuging of 40 ml of the broth at 3200g for 40 min. Only 40 ml were used in order to avoid cell aggregates present in the bottom of the flasks. Cells were washed twice with 8 ml of molecular-grade water and once with 2 ml of 0.5 mM EDTA to remove externally bound metal ions. Biofilms were resuspended in 5 ml of molecular-grade water and washed as with the planktonic cells. The final cell pellets were digested for 1 hour at 100°C in 200 μl of mineral-free concentrated nitric acid (OPTIMA, Fisher Scientific). After dilution with ultra-pure, mineral-free water and centrifugation at 13,000xg to remove any remaining particulates, samples were analyzed by inductively coupled plasma with optical emission spectrometry ICP-OES (7,100 DV; Perkin-Elmer, Waltham, MA) as described in Cobine *et al.* (2013) with simultaneous measurements of S and Zn. As controls, blanks of nitric acid were digested in parallel. Metal concentrations were determined by comparing intensities to a standard curve created from certified metal standards (Spex; Metuchen, NJ). Results were expressed using sulfur (S) measurements as internal denominators representing the amount of cells present in the samples. This experiment was performed once.

EPS quantification in biofilms

EPS production was evaluated by a phenol-sulfuric acid method (Dubois et al., 1951). Seven-day-old *X. fastidiosa* WT, *zur*, and *czcD* cells were collected from PW or PW + kanamycin solid media and were used to inoculate 250 ml flasks containing 50 ml of PD2 or PD2 amended with 400 μM $ZnSO_4$. Flasks were incubated at 28°C and 150 rpm for 5 days, with five replicates per media-strain combination. The planktonic fraction of each flask was

discarded and biofilm resuspended in 5 ml PBS and thoroughly homogenized. A 200 µl aliquot of each biofilm suspension was used for DNA extraction using a modified CTAB method as described above and total cells were quantified by quantitative PCR (qPCR). Two aliquots of 2 ml were centrifuged at 11000x*g* for 30 minutes. The supernatant was transferred to a new tube, and 25 µl of saturated KCl and 4 volumes of ethanol were added. Samples were incubated overnight at 4°C and centrifuged at 3200x*g* for 20 min. EPS precipitates were resuspended in water and total sugars were measured as previously described (Dubois et al., 1951). This experiment was performed once.

qPCRs were conducted using the previously described HL primers and TaqMan® probe set (Francis et al., 2006). Briefly, reactions (20 µl) consisted of 1X ABsolute Blue QPCR ROX mix (ABgene-Thermo Fisher Scientific, Surrey, United Kingdom), 0.4 µl probe (labeled 5'-6-carboxyfluorescein, 3'-Black Hole Quencher-1®, 6-FAM™-BHQ-1), 0.2 µl each primer, and 1 µl DNA. Samples were amplified using an ABI 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA) with the following cycling parameters: 95°C for 1 min, followed by 40 cycles of 95°C for 15 s and 60° C for 1 min. As previously described (De La Fuente et al., 2013), a four-point standard curve was amplified alongside each set of qPCR reactions for sample quantification. This experiment was performed once.

Greenhouse virulence trials.

Nicotiana tabacum 'Petite Havana SR1' (tobacco) seeds (Plant Introduction (PI) number 552516) were obtained from the USDA Germplasm Resources Information Network (GRIN) and germinated in Sunshine Mix #8 (Sun Gro Horticulture Canada Ltd., Vancouver, Canada). Greenhouse temperature was maintained between 20–25°C, and natural sunlight was used. After one month, 40 seedlings were transplanted into 4.5" square pots. One week after

transplanting, plants were fertilized with a slow release fertilizer (Osmocote® Outdoor & Indoor Smart-Release® Plant Food, The Scotts Company, Marysville, OH).

At three weeks post-transplant, tobacco plants were prepared for inoculation as previously described (Francis et al., 2008; De La Fuente et al., 2013) by cutting the top of the stem so that only three healthy adult leaves in the lower portion of the plant remained, numbered 1–3 from bottom to top. Bacterial inoculum was prepared from seven-day-old *X. fastidiosa* WT, *zur*, and *czcD* strains grown in PW or PW + kanamycin solid media. Bacteria from two plates were scraped off and resuspended in succinate-citrate phosphate buffer (Minsavage et al., 1994) to a final $O.D._{600nm}$ of 0.25. A 1 ml tuberculin syringe with a 23 gauge needle was used to inject the plants with 20 µl inoculum per each remaining petiole (three per plant), near the axils. Ten plants were inoculated with each of the strains and ten plants were injected in the same manner with buffer only (mock inoculated). After one week, the inoculation process was repeated in the same leaves originally inoculated.

Plants continued growing from the site where the stem was cut (leaves numbered 4–11). Forty days after the first inoculation (time point 1), five random plants from each of the strains or mock inoculated plants were collected and leaves 4, 7, and 11 used for further analysis.

Bacterial populations in tobacco leaves were quantified via qPCR, as described above. A portion of the lower petiole (50–200 mg) of each leaf was excised, and the weight of the tissue recorded. DNA from each tissue sample was extracted using the CTAB method described above. CFU values determined by qPCR were divided by the previously recorded weight of leaf tissue to determine $CFUmg^{-1}$ leaf tissue. Sixty days after the first inoculation

(time point 2), the remaining plants (five per each strain/buffer) were collected, symptoms rated, and bacterial populations in leaves 4, 7, and 11 determined as above. For data analysis, the $CFUmg^{-1}$ of leaves 4, 7, and 11 were summed as an indicator of the total bacterial population per plant. This experiment was performed once.

Ionome characterization of inoculated plants

At each time point, after excising the petioles, the fully expanded leaves (4 and 7), were prepared for ionome analyses (according to De La Fuente et al., 2013) by drying at 65°C for 1 hour. The whole leaves were then crushed to a fine powder by a plastic mortar and pestle and sampled at 5 and 10 mg of dry weight. Samples were digested for at 1 hour at 100°C in 200 µl of mineral-free concentrated nitric acid (OPTIMA, Fisher Scientific). After dilution with ultra-pure, mineral-free water and centrifugation at 13,000xg to remove any remaining particulates, samples were analyzed by ICP-OES as described in Cobine *et al.* (2013), with simultaneous measurement of Ca, Co, Cu, Fe, K, Mg, Mn, Mo, Na, P, S, and Zn. As controls, blanks of nitric acid were digested in parallel. Mineral concentrations were determined by comparing emission intensities to a standard curve created from certified mineral standards (SPEX CertiPrep, Metuchen, NJ). Repeated analysis of different amounts (5 and 10 mg) of individual samples showed less than 5% variation. For data analysis, the mineral concentrations determined for leaves 4 and 7 were summed as an indicator of the total mineral content of the plant. This experiment was performed once.

Statistical analyses

Data from biofilm production, intracellular Zn accumulation, EPS production, and bacterial colonization of tobacco was analyzed by ANOVA and means compared by Fisher's LSD test. For the analysis of the ionome of inoculated plants, each mineral element was analyzed

separately by ANOVA and means compared by Fisher's LSD test. The percentage of symptomatic leaves was subjected to the following transformation: arcsine [SQRT (x)] and analyzed as described before. Least-significant differences were compared at a level of significance of $P \leq 0.05$. All analyses were conducted with SAS 9.2 (SAS Institute Inc. NC).

Results

Bacterial growth in media with different Zn concentrations

When grown in 96-well plates in PD2, all bacterial strains, *X. fastidiosa* WT and the *zur* and *czcD* mutants showed the same overall growth kinetics. *zur* and *czcD* showed a transitory increase in $O.D._{600nm}$ between five and six days post inoculation (dpi). By seven dpi, $O.D._{600nm}$ of all strains was similar (Figure III-1A). Biofilm production of *zur* (measured seven dpi), was higher than in the WT, biofilm production in *czcD* was not different from WT strain (Figure III-1B).

When PD2 was supplemented with different amounts of $ZnSO_4$, both *zur* and *czcD* strains showed reduced growth compared to the WT. When $ZnSO_4$ was added to a final concentration of 25 μM, by seven dpi, $O.D.s_{600nm}$ of the WT cultures reached values twice as high as those of *czcD* cultures and three times higher than those of *zur* cultures (Figure III-1C). Biofilm production in this medium was highest for *zur* cultures, *czcD* did not differ from the WT strain for biofilm production (Figure III-1D).

When $ZnSO_4$ was added to a final concentration of 100 μM, only the WT was able to grow, while *zur* and *czcD* mutants failed to grow (Figure III-1E). Biofilm production was highest for the WT strain; *zur* showed decreased biofilm production with respect to the WT strain and *czcD* showed decreased biofilm with respect to the WT and *zur* strains (Figure III-1F).

When $ZnSO_4$ was added to a final concentration of 400 μM, none of the strains showed any growth (Figure III-1G). Biofilm production was much reduced and was equal for all three strains (Figure III-1H).

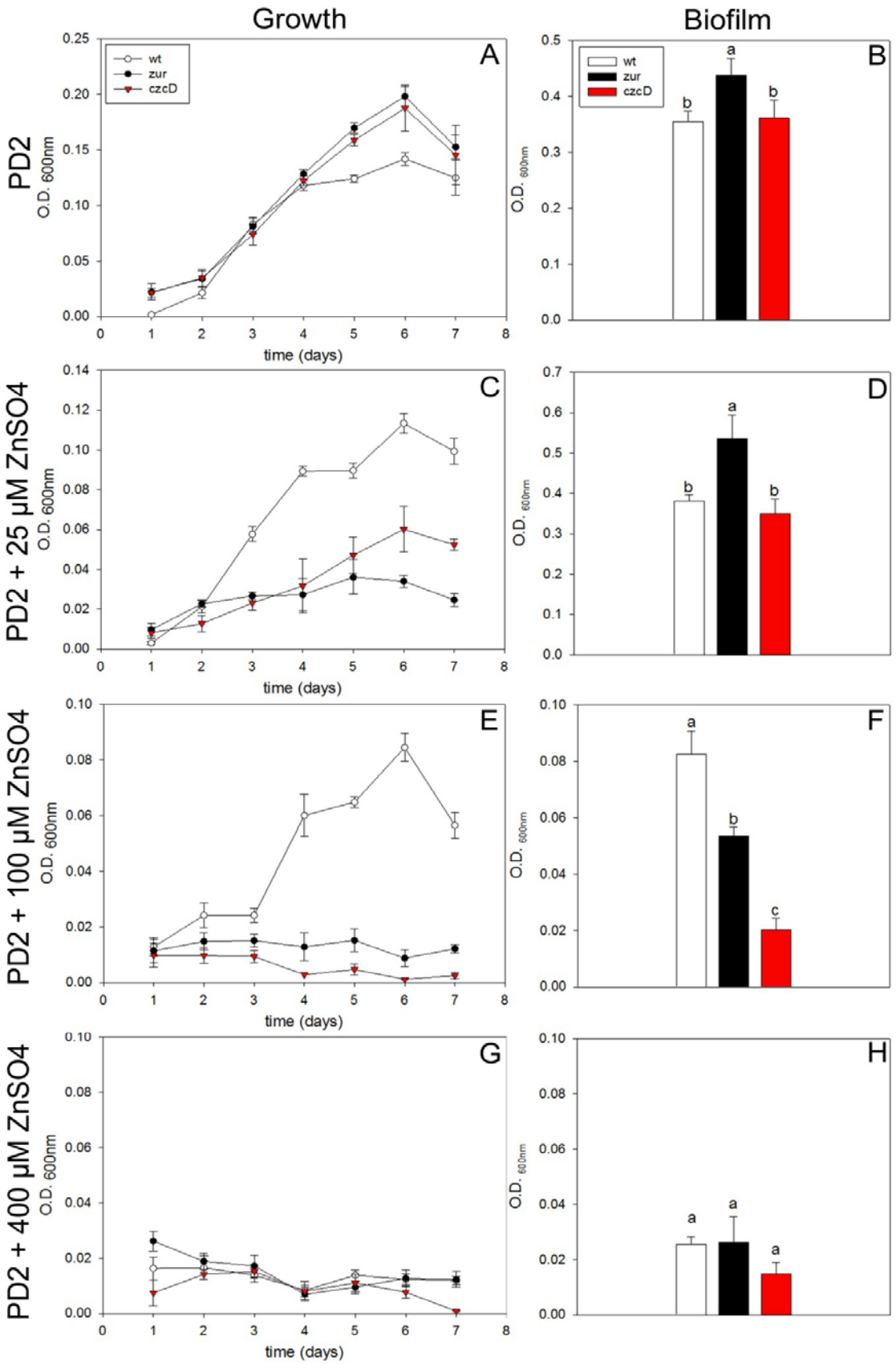
Growth
Biofilm
PD2
PD2 + 25 µM ZnSO4
PD2 + 100 µM ZnSO4
PD2 + 400 µM ZnSO4
A
B
C
D
E
F
G
H
wt
zur
czcD
O.D. 600nm
time (days)
a
b
c

Figure III-1. Growth and biofilm production of mutant strains in media supplemented with Zn.

X. fastidiosa WT, *zur* and *czcD* were incubated in 96-well containing PD2 supplemented with different amounts of $ZnSO_4$ for seven days at 28°C and 150 rpm. Bacterial growth (A, C, E, G) was recorded daily and biofilm production was evaluated at seven dpi by crystal violet assay (B, D, F, H). Data represents mean standard error (n=6).

In 50% SAP, the growth of *zur* and *czcD* was much reduced compared to the WT; cultures of this strain reached $O.D._{600nm}$ three times higher than those of any of the mutant strains (Figure III-2A). *zur* and *czcD* also showed reduced biofilm production with respect to the WT (Figure III-2A). None of the strains grew in SAP (Figure III-2). Also in this medium, biofilm production was much reduced and was equal for all three strains (Figure III-2).

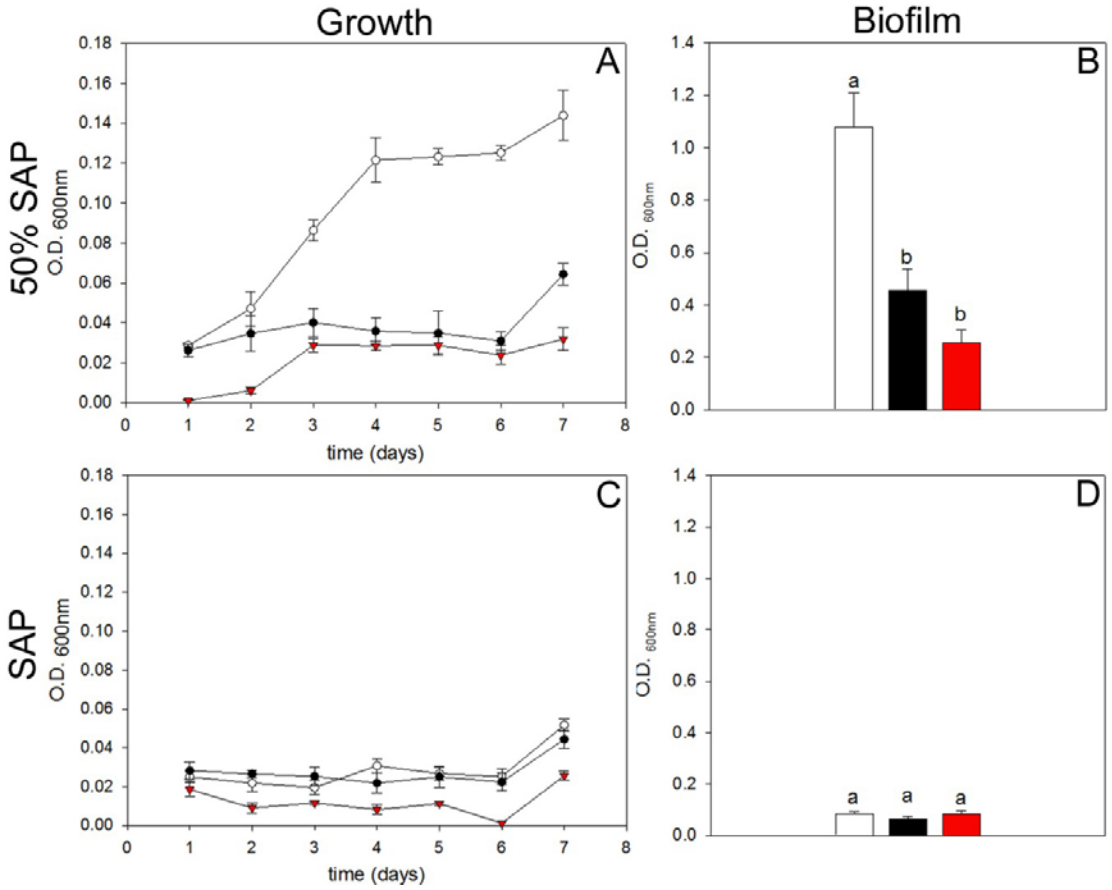

Figure III-2. Growth and biofilm production of mutant strains in xylem sap**.**

X. fastidiosa WT, *zur*, and *czcD* were incubated in 96-well plates for seven days at 28°C and 150 rpm in 50% SAP (A, B) or SAP (C, D). Bacterial growth (A, C,) was recorded daily and biofilm production was evaluated at seven dpi by crystal violet assay (B, D). Data represents mean ± standard error (n=6).

Bacterial zinc accumulation

For all strains, intracellular accumulation of Zn was higher in biofilm compared to planktonic cells and both, *zur* and *czcD* showed increased Zn accumulation compared to the WT in both phases (Figure III-3). For planktonic cells, all strains had different levels of Zn accumulation.

The highest Zn accumulation was seen for *czcD* (0.048 ±0.001 g Zn/ g S), followed by *zur* (0.036±0.002 g Zn/ g S) and the WT *X. fastidiosa* (0.029±0.002 g Zn/ g S)(P<0.05).

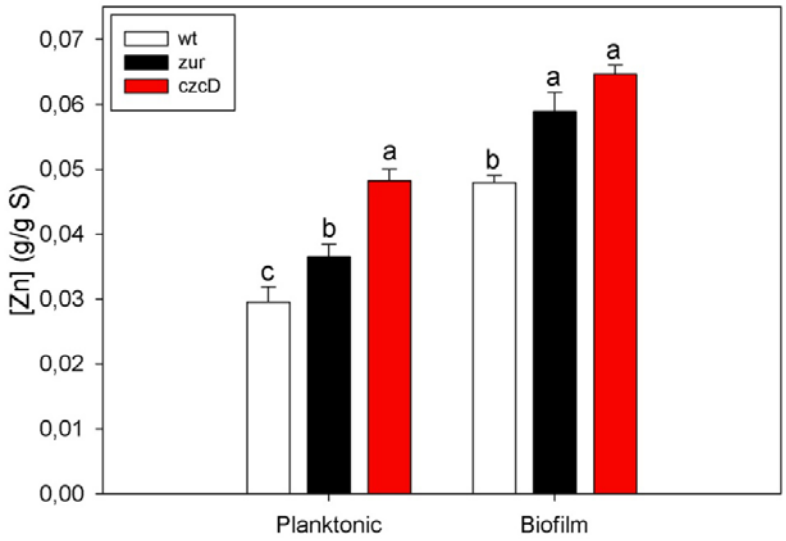

Figure III-3. Bacterial zinc accumulation**.**

X. fastidiosa WT, *zur* and *czcD* were grown in 50 ml of PD2 at and 150 rpm for three days and the $ZnSO_4$ concentration was adjusted to 100µ M. Cultures were further incubated overnight and intracellular Zn content was evaluated by ICP-OES as described in materials and methods. Data represent mean ± standard error (n=5).

In the case of biofilm cells, both *zur* and *czcD* showed increased intracellular Zn accumulation compared to the WT)(P<0.05) strain but they did not differ from each other (P>0.05). Zinc accumulation was 0.058±0.003 g Zn/ g S for *zur*, 0.0064±0.001 g Zn/ g S for *czcD* and 0.047±0.001 g Zn/ g S for the WT strain.

EPS production

To determine whether the mutations in *zur* and *czcD* have any effect on EPS production, this trait was measured in biofilms of the WT, *zur* and *czcD* strains grown at different concentrations of $ZnSO_4$. *zur* and *czcD* mutants had reduced EPS production only at high $ZnSO_4$ concentrations (Table III-3). EPS production in both mutants (*zur*: 4.78e-7 ± 1.4e-7; *czcD*: 3.58e-7 ± 9.6e-8 µg glucose eq. $cell^{-1}$) was approximately half of what was observed for the WT (1.18e-6 ± 7.3e-7 µg glucose eq. $cell^{-1}$) ($P<0.05$). In the non-Zn-supplemented media, the three strains did not differ in the amount of EPS produced (wt: 1.40e-10 ± 3.7e-11; *zur*: 1.47e-10 ± 2.1e-11; *czcD*: 6.94e-10 ± 5.1e-10) ($P>0.05$).

Table III-2. Effect of zinc concentration on EPS production of mutant strains.

	EPS (µg glucose eq. $cell^{-1}$)	
Strain	**PD2**	**PD2+400 µM $ZnSO_4$**
wt	1.40 ± 0.37 e-10 a	1.18 ± 0.73e-6 a
zur	1.47 ± 0.21 e-10 a	4.78 ± 1.4e-7 b
czcD	6.94 ± 5.1e-10 a	3.58 ± 0.96e-7 b

Data are the mean ± standard error (n=5); different letters indicate significant differences within each column ($P< 0.05$).

Virulence characterization

The virulence of *zur* and *czcD* mutants was tested by needle inoculation of tobacco plants grown in the greenhouse. Bacterial growth was assessed 40 days post inoculation, when plants where completely asymptomatic, and 60 days post inoculation, when symptoms were beginning to develop. For all the strains, 100% of the inoculated plants were colonized by *X. fastidiosa* as detected by qPCR.

At 40 days post inoculation, *zur* showed reduced colonization of the infected plants compared to the WT. The bacterial population detected for this strain in infected plants (6.1± 1.4 log CFU mg^{-1}) was approximately 100-fold lower than that of the WT strain (7.8±0.18 log CFU mg^{-1}) ($P<0.05$). On the contrary, for the *czcD* mutant the bacterial population detected in planta (7.78±0.10 log CFU mg^{-1}) did not differ from that of the WT ($P>0.05$) (Figure III-4). At 60 days post inoculation, both mutant strains showed reduced populations in planta compared to that of the WT ($P<0.05$). Bacterial populations detected in tobacco plants were 8.37±0.34 and 8.61±0.18 log CFU mg^{-1} for plants inoculated with *zur* and *czcD*, respectively. The bacterial population detected in tobacco plants inoculated with the WT was 9.17 ±0.08 CFUmg^{-1} (Figure III-4). This represents, approximately, 10- and 5-fold decreases in bacterial populations in planta for *zur* and *czcD* compared to the WT, respectively.

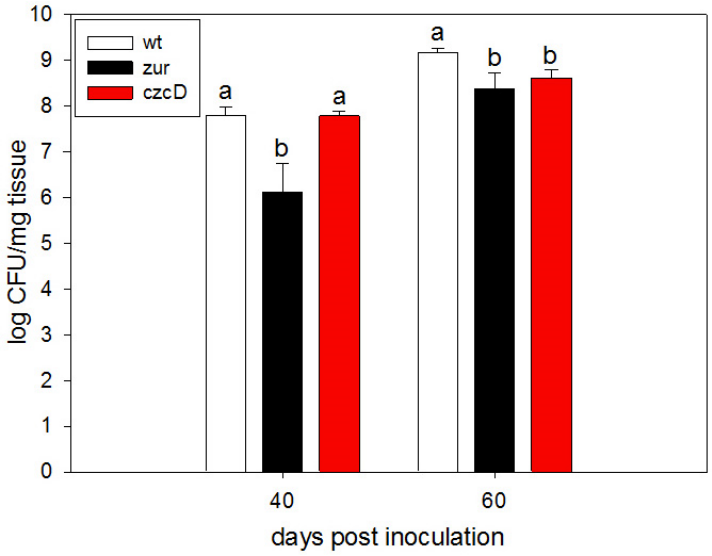

Figure III-4. Bacterial colonization of tobacco.

Tobacco plants were needle inoculated with *X. fastidiosa* WT, *zur*, and *czcD* and maintained in the greenhouse for 60 days. Bacterial populations were quantified from leaf petioles of

inoculated plants by qPCR at 40 and 60 dpi. Data represent means ± standard error (five plants per strain, three leaves per plant).

Foliar symptoms rated at 60 days post inoculation included: chlorotic lesions, necrotic lesions, and wilting. Plants inoculated with both mutant strains showed reduced symptom development compared to the WT. Plants inoculated with *zur* showed the least severe symptoms. Wilting and necrotic lesions were not detected in these plants and only 7±5% of leaves/plant showed chlorotic lesions compared to 50±10% in the WT-inoculated plants (Figure III-5). Plants inoculated with the *czcD* strain did not show necrotic lesions and the percentage of wilted leaves/plant (15±5%) was significantly lower than that of the WT-inoculated plants (52±12%) ($P<0.05$).The percentage of leaves/plant showing chlorotic lesions (25±14%) did not differ from that of the WT-inoculated plants (50±10%) ($P>0.05$) (Figure III-5).

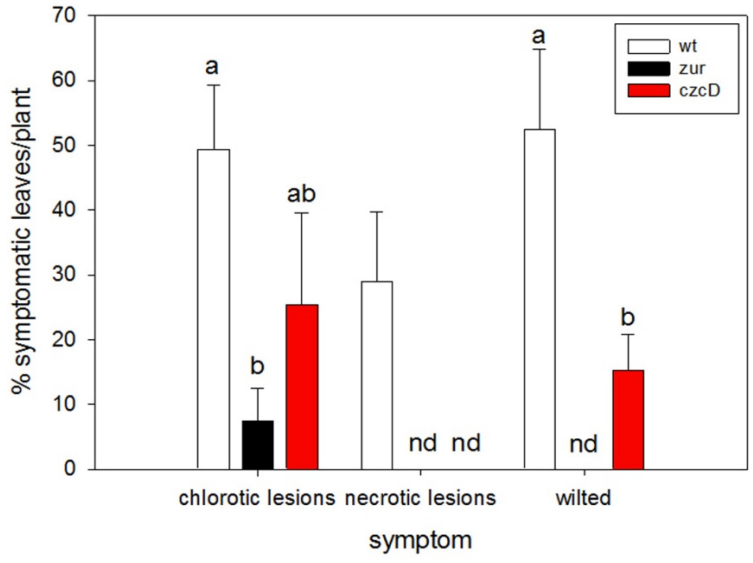

Figure III-5. Disease severity**.**

Tobacco plants were needle inoculated with *X. fastidiosa* WT, *zur* and *czcD* and maintained in the greenhouse for 60 days. Symptoms were rated 60 dpi. Data represent means ± standard error (five plants per strain). nd= symptoms were non-detected.

Ionome characterization of inoculated plants

The leaf ionome of infected plants was analyzed as an indication of the physiological status of the whole plant, to assess the effects of bacterial infection on the host. At 40 days post inoculation, significant differences in the mineral content of *X. fastidiosa*-inoculated plants with respect to the mock-inoculated plants were observed for Ca, K, Mg, Na, Ni, and P. Plants inoculated with the WT showed increased levels of Ca, K, Mg, Na, Ni, and P that varied from 25 to 55% depending on the mineral element (Figure III-6A)(P<0.05). Plants inoculated with *czcD* showed increased levels of K only. K levels were 25% higher than in the mock inoculated plants (P<0.05) and were not different than the K levels of WT inoculated plants (P>0.05). Plants inoculated with *zur* did not show any significant difference in mineral composition with respect to the mock inoculated plants (P>0.05) (Figure III-6A).

At 60 days post inoculation, significant differences in the mineral content of *X. fastidiosa*-inoculated plants with respect to the mock-inoculated plants were observed for B, Ca, K, Mg, Na, P, and S. Plants inoculated with the WT showed increased levels of B, Ca, Mg, Na, and S that varied from 25 to 60% depending on the mineral element (Figure III-6B) (P<0.05). Plants inoculated with *czcD* showed increased levels of Mg (~ 35%) and decreased levels of K (~ 40%) only (P<0.05). Plants inoculated with *zur* only showed a decrease in the levels of P with respect to mock-inoculated plants. P levels were 40% lower than those of the mock-inoculated plants (Figure III-6B) (P<0.05).

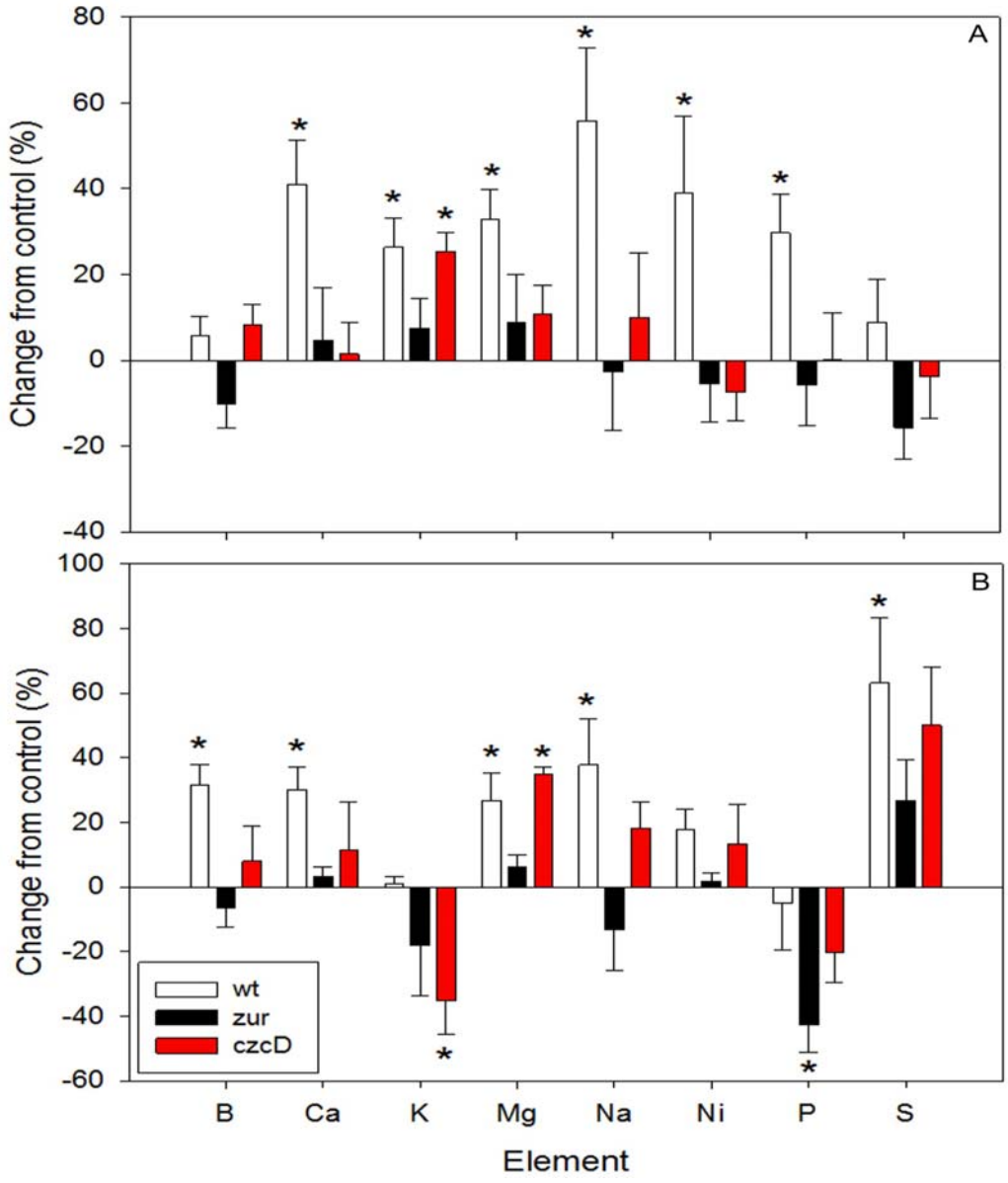

Figure III-6. Leaf ionome of inoculated tobacco plants.

Tobacco plants were needle-inoculated with *X. fastidiosa* WT, *zur* and *czcD* and maintained in the greenhouse for 60 days. The ionome of leaves 4 and 7 (see materials and methods) was characterized by ICP-OES at 40 dpi (A) and 60 dpi (B). Mean relative percentage of change and standard errors were calculated by comparing leaf element concentrations (ppm) between leaves of *X. fastidiosa*-inoculated plants vs. leaves of mock-inoculated plants. Data represents mean ± standard error (n=5). *

indicates significant difference with respect to the mock-inoculated plants ($P<0.05$). Metal elements not shown were not significantly different from the control plants for any of the time point and strains tested.

Discussion

In the present study, the requirement of *zur* and *czcD* for full virulence of *X. fastidiosa* was established. These genes are required for growth in media with high Zn concentration as well as in xylem sap. Both mutants exhibited increased intracellular accumulation of Zn compared to the WT, indicating that these genes are involved in Zn efflux in *X. fastidiosa*. Deletion of either of these genes causes a reduction in the ability of *X. fastidiosa* to colonize a model plant host and cause disease, with the effect of the *zur* mutation being more pronounced than the mutation in *czcD*. Since Zn is less concentrated in PD2 (2.8±0.7 μM) that in xylem sap (38±5 μM) (Cobine *et al*., 2013), the results suggest that the ability to tolerate high Zn concentrations correlates with the ability to grow in xylem sap and with bacterial virulence in planta. *zur* and *czcD* mutants also showed decreased EPS production when grown at high Zn concentrations, which may be, in part, responsible for the observed reduction in virulence. Additionally, neither of the mutants was able to induce an extensive alteration of the host leaf ionome at any of the time points analyzed, as was observed for the WT *X. fastidiosa*, which indicates that they are much reduced in their ability to alter the host physiology. All the experiments are being currently repeated to confirm the reproducibility of the results.

The zinc uptake regulator, Zur, is a transcriptional regulator of the fur family, which at high Zn^{2+} concentrations binds to the promoter region of the *znu*ABC Zn-uptake system to repress its transcription (Patzer & Hantke, 1998) and to the promoter of *czcD* to promote its

transcription (Huang & Tang, 2008). Additionally, *zur* is known to regulate the expression of genes involved in iron uptake (*tonB*), bacteriocin production, multidrug detoxification (*tolC*) and oxidative stress resistance (He et al., 2007). Regulation of virulence by *zur* has been observed in several bacteria. *zur* deletion causes reduction in virulence in the animal pathogens *Salmonella typhimurium* and *Pseudomonas aeruginosa* (Campoy et al., 2002, Wang et al., 1996). In bacterial species closely related to *X. fastidiosa*, *X. campestris* pv. *campestris* and *X. oryzae* pv. *oryzae,* mutations in *zur* caused reductions in virulence (Tang et al., 2005, Yang et al., 2007), which were attributed to a reduction in EPS production (Tang et al., 2005) or reductions in both EPS production and tolerance to oxidative stress (Yang et al., 2007). *czcD* is a member of the Cation Diffusion Facilitator (CDF) family that localizes to the inner membrane and is required for Zn efflux (Nies, 2003, Huang & Tang, 2008). Expression of *czcD* is up regulated by high Zn levels in *Streptococcus pneumonia* (Jacobsen et al., 2011). This gene is also upregulated during infection of host cells by *Brucella melitensis* (Rossetti et al., 2011).

The results presented here show that the degree of tolerance to Zn correlated with bacterial populations in planta. This suggests that the reduction in virulence observed for both mutants is likely due to the reduction in tolerance to high zinc levels. Unexpectedly, bacterial accumulation of Zn did not correlate with susceptibility to Zn in the growth medium, since *zur*, the strain most sensitive to Zn, accumulated less Zn than *czcD*. This suggests that Zn uptake is also affected in the *zur* mutant. Since *zur* is a transcriptional regulator, it is not surprising that its deletion affects many traits. All of the previously mentioned traits that are known to be regulated by *zur* in other bacteria are important for virulence in *X. fastidiosa*. Bacteriocin production (Simpson et al., 2000), iron uptake through *tonB* (Cursino et al., 2009), and multidrug detoxification through *tolC* (Reddy et al., 2007) are required for full

virulence in this bacterium. In addition, the transcription of arginine uptake genes is regulated by *zur* in *Bacillus thuringiensis*, and the levels of this amino acid are increased in *X. fastidiosa*-infected citrus trees (Purcino et al., 2007). Thus, it could be speculated that arginine is used as a carbon and/or nitrogen source by this bacterium. It would be interesting to determine if *zur* controls the expression of any of these genes in *X. fastidiosa*. The regulatory nature of *zur* may also explain why this mutant has increased biofilm production compared to the WT despite the fact that Zn supplementation reduces biofilm production (Cobine et al., 2013). This effect might be related to production of adhesins. In *Streptococcus pneumonieae*, AdcR, the Zn transcriptional regulator in this bacterial group, controls the expression of adhesins required for adherence to the respiratory mucosa of the host (Elsner et al., 2002, Panina et al., 2003, Shafeeq et al., 2011). Expression analyses of adhesins as well as assessment of attachment strength of the *zur* mutant would be important to elucidate how this gene regulates biofilm production.

Both *zur* and *czcD* mutants showed a reduced alteration of the host ionome compared to the WT. Studying the host leaf ionome, defined as the mineral nutrient and trace elements found in an organism, allows for evaluation of the physiological status of the whole plant (Lahner et al., 2003, Baxter et al., 2008). This approach is especially relevant for the study of *X. fastidiosa*-plant interactions, since this bacterium inhabits the xylem vessels, through which mineral elements are transported to the leaves, and has been shown to modify the host ionome (De La Fuente et al., 2013).

There are no published studies that analyze the leaf mineral composition of *X.fastidiosa*-infected plants prior to symptom appearance. A similar study using tobacco plants (De La Fuente et al., 2013) analyzed the leaf ionome of infected plants but it corresponded to a pool

of five different time points, with sampling pre and post-symptom appearance; time points were analyzed separately for Ca and P only. On the contrary, leaf ionome results presented here for the second time point, when symptoms are developed, in general agree with previous studies. Ca has been found to be increased in inoculated tobacco plants (De La Fuente et al., 2013), Ca and Mg were increased in infected grapevines (Goodwin et al., 1988) and Na was increased in infected peaches (Andersen & French, 1987). Also a decrease in K levels has been found in infected grapevines, peaches and citrus (Goodwin et al., 1988, Andersen & French, 1987, Silva-Stenico et al., 2009).

Ca has several functions in the plant, such as contributing to the rigidity of cell membranes and primary cell walls and interacting with hormones involved in cell elongation (Pilbeam & Morley, 2007). On the other hand, it is known to promote the expression of many virulence traits in *X. fastidiosa* (Cruz et al., 2012). Thus, the reason for this increased Ca accumulation in the plant is not clear, and experiments published so far do not allow drawing conclusions in this matter. Results shown here demonstrate that only the fully virulent WT strain was able to induce an increase of Ca accumulation in the host plant at both time points analyzed. *zur* and *czcD* mutants, which had decreased population in planta and induced less severe symptoms, were not able to induce accumulation of Ca in tobacco leaves. This suggests that the accumulation of Ca in the plant is induced by the bacterium as a virulence strategy. It can be hypothesized that *X. fastidiosa*, through an unknown mechanism, induces an increase in Ca uptake that is used by the bacterium to improve its colonization abilities, and strains that fail to induce this response have compromised performance in planta.

Many of the observed changes in leaf ionome observed here are in agreement with the responses observed in plants experiencing water stress and decreased photosynthetic rate, which are known responses of *X. fastidiosa* infected plants (Choi H. K., 2013, McElrone A.

J., 2004). The alterations in the accumulation of K, Na, Mg and S observed here fit into the water stress-photosynthetic damage scenario.

K is the main osmolite in plants and its level usually correlates with the water status of the plant (Mengel, 2007). Here, leaf potassium levels were increased before symptom development in plants inoculated with the WT and the *czcD* mutant with respect to the mock-inoculated plants. At this time point, populations of both strains did not differ from each other and were 100-fold higher than the population of the *zur* mutant. Thus, *X. fastidiosa* seems to need to reach a certain population minimum to trigger this response in the plant. The increase in K might be involved in osmotic readjustment. Some plants accumulate K in the initial phases of water stress because osmotic adjustment through ion uptake is more energetically efficient than synthesis of organic solutes (Hu & Schmidhalter, 2005). On the contrary, after symptom appearance, K levels tended to be reduced compared the first time point. Depressed K accumulation is a well-known characteristic of water stress in plants (Datnoff et al., 2007). However, only the plants inoculated with *czcD* had decreased K levels compared with the mock inoculated plants. The significance of this fact is not clear. Na appears to be acting in osmotic readjustment too. This metal can replace K as an osmolite in some situations and at low doses it can improve water balance due to increased stomatal closure (Marschner, 1995). In addition, increases in the levels of Mg have been found in water stressed grapevines (Andersen P.C., 1985). Sulfur accumulation has not been reported previously in *X. fastidiosa* infected plants. However, the great majority of S in the cell is in the form of glutathione (Marschner, 1995), and up regulation of genes involved in glutathione metabolism has been observed after symptom appearance and in water stressed plants, presumably to alleviate oxidative damage in the photosynthetic apparatus (Choi H. K., 2013, Shinozaki & Yamaguchi-Shinozaki, 1997). Glutathione, also needs Mg for its activity (Marschner, 1995),

thus an increase in glutathione would also help explain the observed increased levels in of Mg. Most of these responses, increased accumulation of K at the first time point, increased accumulation of Mg and Na, and increased accumulation of S at the second time point were only significant in the WT inoculated plants, suggesting that these plants suffer a more pronounced water stress compared to plants inoculated with the *zur* or *czcD* mutants.

Ni accumulation in *X. fastidiosa* infected plants has not previously been reported. However, this bacterium is known to affect nitrogen metabolism in the host (Purcino et al., 2007; Choi H. K., 2013) and Ni has only one known function in plants: it is a cofactor of urease, an enzyme that catalyzes the production of ammonia from urea (Datnoff et al., 2007). Thus, the increased accumulation of Ni observed in infected plants at the first time point, prior to symptom development, may be related to the altered mobilization of nitrogen in infected plants. Only the WT appears to be capable of inducing this response in the host which suggests that both mutants have a reduced capacity of utilization of the host nitrogen.

The levels of P, and B of infected plants observed here, differed from those found in other studies (De La Fuente et al., 2013, Andersen & French, 1987). In particular, the levels of P in tobacco have been shown to decrease as the infection progresses (De La Fuente et al., 2013). However, P levels in this study were increased before symptom appearance in WT inoculated plants and were not significantly different from the mock inoculated plants in the second time point. This suggests that the accumulation of this mineral element in response to *X. fastidiosa* infection, and perhaps the accumulation of others such as B, are more variable and subject to different conditions such as plant host species, time of the year and fertilization.

Importantly, the concentration of a certain nutrient in the plant can affect the concentrations of others. For example, increased Ca supply to the roots promotes K uptake (Marschner, 1995). The present experimental design does not account for these effects; thus, which alterations in the plant mineral composition are a direct effect of *X. fastidiosa* infection cannot be attributed with certainty.

Overall, the results presented here show that detoxification of Zn in the host is an important virulence strategy for *X. fastidiosa*. Additionally, this process might be coupled with the regulation of other virulence factors in this pathogen. Alteration of the host leaf ionome, at least in regards to increased Ca levels, appears to be a virulence strategy employed by this bacterium to better colonize the host, and mutants affected in Zn detoxification fail use this virulence mechanism. In addition, these mutants fail to induce physiological changes associated with water stress and disruption of nitrogen metabolism in the host plant. In the long term, this information could be used for disease management. Selection of sites and/or rootstocks with high Zn and low Ca levels/uptake might be useful to create conditions that restrict disease development.

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Chapter IV
General conclusions

This works describes the responses of the bacterial plant pathogen *Xylella fastidiosa* to zinc, a heavy metal present in xylem sap of the host plant, the bacterium's natural environment.

Zinc has a pleiotropic effect on *X. fastidiosa*. Increasing the levels of this metal in the growth medium prevents growth and production of biofilm. Moreover, it is suggested in this study that *X. fastidiosa* can enter the viable but non-culturable state (VBNC), and that Zn can induce an early onset of this state. Despite the negative effects of Zn on growth and survival of *X. fastidiosa*, this metal induces a pronounced increase in the production of exopolysaccharide (EPS), an essential pathogenicity factor in this bacterium, and the formation of compact and firmly attached aggregates under after reinoculation events in microfluidic chambers. Taken together, the results suggest that high Zn concentration in the growth medium stresses *X. fastidiosa* and that it responds, in part, by increasing the production of EPS.

Zinc, at the levels present in the plant host, constitutes a preformed barrier that limits the spread of this bacterium in the xylem tissues and delays the production of disease symptoms. Correct Zn homeostasis is required for regulation of biofilm and EPS production in *X. fastidiosa* as well as for the induction of responses associated with water stress in the plant host. Thus, detoxification of toxic levels of Zn, constitute an important virulence mechanism for *X. fastidiosa.*

Importantly, the results presented here suggest that the previously observed accumulation of calcium in plants infected with *X. fastidiosa* constitutes a virulence strategy. Strains that fail to induce the plant accumulation of Ca have restricted colonization abilities and induce less severe symptoms in the host.

The work presented here reinforces the important role that mineral elements have in *X. fastidiosa*-host interactions and suggest that the mineral status of the host plant could be manipulated as a disease management strategy.