Applied and Basic Aspects of Copper Interaction with *Xylella fastidiosa*

by

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Abstract

Xylella fastidiosa, a gram-negative, xylem-limited plant pathogenic bacterium that causes severe diseases and asymptomatic colonization in more than 600 different plants worldwide, including many economically important crops. Great economic impact and job losses are caused by this pathogen, therefore broad attention was drawing from science and society. Copper (Cu) as a widely used antimicrobial agent is hardly used to control *X. fastidiosa* in the field, but is often applied to its hosts for other pathogens' management. High Cu accumulations in field soils were found in *X. fastidiosa* living environment and threatened its survival. In my dissertation, I attempted to understand the interactions between Cu and *X. fastidiosa*, especially in aspects of *X. fastidiosa* virulence, molecular basis of Cu resistance/homeostasis, and further features that may influence Cu resistance ability in *X. fastidiosa*.

First, I conducted in planta experiments to determine the influence of Cu in *X. fastidiosa* infection using tobacco as a model. Uptake of Cu was noticed in plants treated with $CuSO_4$-amended water. However, this did not help to control disease symptoms development, nor the growth of *X. fastidiosa* in planta. Then, molecular basis of Cu resistance/homeostasis was studied in relation to virulence. Mutants of Cu-related genes *copA*, *copB* and *cutC* were more sensitive to Cu than the wild-type (WT) strain. All the mutants increased disease severity under Cu-amended conditions, and the increments were higher than WT. Last, our analysis showed that no clear correlation was found between Cu resistance/homeostasis ability and *X. fastidiosa* phylogeny, host and/or location of isolation.

In general, I found that Cu content in plant could influence virulence of *X. fastidiosa*. Cu resistance/homeostasis in *X. fastidiosa* is important for its in planta colonization and disease

symptoms development, which is associated with *copA*, *copB* and *cutC* genes that function differently to fulfill Cu resistance/homeostasis. Meanwhile, Cu resistance/homeostasis in *X. fastidiosa* is not simply decided by host and/or location of isolation. My research help to unveil the mechanisms of Cu resistance/homeostasis in *X. fastidiosa*, and highlight the important role that Cu plays in *X. fastidiosa* full virulence as well as its interaction with host plants.

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Xylella fastidiosa

Xylella fastidiosa is a fastidious, gram-negative, plant pathogenic bacterium that only lives in plant xylem vessels and foreguts of sap-feeding insects (Chatterjee et al. 2008; Redak et al. 2004; Wells et al. 1987). It was originally considered as an obligate aerobe but is more likely to be a facultative anaerobe based on recent studies (Shriner and Andersen 2014; Wells et al. 1987). The cell has a rod shape, with no flagella but two types of pili (type I and type IV) attached to one of the cell's pole (Janse and Obradovic 2010). Based on taxonomic classification, *X. fastidiosa* belongs to the order Xanthomonadales, family Xanthomonadaceae, and genus *Xylella* (Wells et al. 1987). It could be further divided into four genetically distinct subspecies: *X. fastidiosa* subsp. *fastidiosa*, *X. fastidiosa* subsp. *multiplex*, *X. fastidiosa* subsp. *sandyi*, and *X. fastidiosa* subsp. *pauca* (Scally et al. 2005; Schaad et al. 2004). Each subspecies has its own host and geographical preferences.

Pathogen Establishment and Symptom Development

X. fastidiosa as a xylem-limited pathogen in plants, could only be transmitted by sap-feeding insects such as sharpshooters, leafhoppers, and spittlebugs (Severin 1949, 1950). During the feeding process of insects, bacteria are injected directly from insects' feeding organ into host plants xylem vessels. This process is different from infection processes of other plant vascular

pathogens, where bacteria infect plant organ cells at first and then move into plant vascular systems (Bae et al. 2015; Chatterjee et al. 2008). Once *X. fastidiosa* gets inside the xylem, sap flow of plants help it moves upward with the flow direction. The twitching movement of *X. fastidiosa*, carried by the extension, tethering and then retraction of polar type IV pili, allows it to move against the sap flow (Mattick 2002; Meng et al. 2005). The horizontal movement of *X. fastidiosa*, from one vessel element to the neighboring vessel elements, is achieved by its ability of dissolving the pit membrane, which is a primary cell wall between neighboring vessel elements (Pérez-Donoso et al. 2010). To successfully establish in host plants, *X. fastidiosa* forms biofilm in host plants xylem vessels, which is a crucial virulence factor of this pathogen. The biofilm formation process starts as bacterial cells attached to the surface of vessel elements, followed by cells reproduction and production of various extracellular compounds. Eventually, the structure of bacterial cells, self-produced EPS (extracellular polysaccharides), and other substances form mature biofilms, which as a big cluster can block xylem vessels (Castiblanco and Sundin 2016; Chatterjee et al. 2008; Newman et al. 2003). Blockage also happens when host plants sense the pathogen and respond to the attack. Plants attempt to stop the spread of pathogens by the production of tyloses, pectin-rich gels and crystals in xylem. This defense process could also occlude vessels (Sun et al. 2013). The blockage of xylem vessels caused by reasons mentioned above influence the water and nutrient transportation in host plants. The deficiencies of water and nutrients, caused by xylem blockage in host plant results in leaf scorch and fruit shriveling that are typical symptoms of *X. fastidiosa*-caused diseases.

X. fastidiosa Caused Worldwide Diseases

X. fastidiosa is found causing severe disease or as an asymptomatic symbiont in around 600 different crops and plants worldwide (Cariddi et al. 2014; EFSA 2020). In the U.S., *X. fastidiosa* was first associated with Pierce's disease of grape (PD), which was found in Anaheim,California in 1892 (Pierce 1892). The disease causes leaf scorch and fruit shriveling, eventually leading to plant death. Since the 1999 outbreak in Temecula, PD draws great attention as it causes huge economic impacts to the wine industry especially in California (Bruening et al. 2014; Hopkins and Purcell 2002). Millions of dollars are lost yearly in the U.S. due to PD (Tumber et al. 2014). The subspecies causing PD is *X. fastidiosa* subsp. *fastidiosa*, which is native to Central America. This subspecies also infects plants like alfalfa, maple and almond, causing alfalfa dwarf, bacterial leaf scorch (BLS) of maple and almond leaf scorch, respectively (Almeida and Purcell 2003; Daugherty et al. 2010; Hartman et al. 1996). BLS of blueberry causes great economic impact to the blueberry industry of southern Georgia and northern Florida, where the disease was originally found. BLS of blueberry can be caused both by the infection of *X. fastidiosa* subsp. *fastidiosa* and subsp. *multiplex* (Chang et al. 2009; Di Genova et al. 2020; Oliver et al. 2015). *X. fastidiosa* subsp. *multiplex* also inflects plants like plum, peach, elm and almond (Janse and Obradovic 2010). In the southern regions of the U.S., *X. fastidiosa* subsp. *sandyi* was found infecting oleander, causing oleander leaf scorch (Schuenzel et al. 2005). In South America, citrus variegated chlorosis (CVC) is an important disease caused by *X. fastidiosa* subsp. *pauca*. The disease causes symptoms of spotty chlorosis on leaves, and fruits of infected plants are small and hard. The citrus industry of Brazil losses around 120 million dollars every year due to the CVC (Goncalves et al. 2012; Pooler and Hartung 1995). This subsp. *pauca* also infects coffee (Almeida et al. 2008). In 2013, olive quick decline syndrome (OQDS), causing by the infection of *X. fastidiosa* subsp. *pauca*, was reported in Italy. Millions of olive trees have been destroyed

in there due to OQDS. Later *X. fastidiosa* was found infecting olive trees in France, Spain and other regions of Southern Europe. OQDS threatens the whole olive industry in Europe, causing billions in economic losses and job losses (Cariddi et al. 2014; Martelli et al. 2016; Rapicavoli et al. 2018; Saponari et al. 2017; Schneider et al. 2020).

Ionome of *X. fastidiosa* and Its Host Plants

Ionome of plants, the mineral elements content of plant, has been used as an indicator of the physical status of plants (Baxter et al. 2008; Lahner et al. 2003). It has also been used to help the study of plant-pathogen interactions (De La Fuente et al. 2013; Nicolas et al. 2019; Oliver et al. 2014). *X. fastidiosa* survives in xylem vessels of plant where mineral elements and water are transported. Minerals in xylem sap provide nutrient sources for the plant and pathogens but depending on the concentration some of them could also be stress factors. Copper (Cu) that acts as a metal cofactor for many enzymes, is necessary for metabolic activity in bacteria. But it needs to be under careful homeostatic regulation, since high cellular concentration leads to toxic effects to cells (Cooksey 1993; Festa and Thiele 2012). The studies carried in *X. fastidiosa* indicate that lower concentrations (0-200μM) of Cu promotes cell growth and biofilm formation. While, high concentrations (>200μM) of Cu inhibits these processes (Cobine et al. 2013). Meanwhile, biofilm cells are more resistant to Cu and accumulates more Cu than planktonic cells (Cobine et al. 2013; Rodrigues et al. 2008). Cu could also induce the production of persister cells in *X. fastidiosa*, which are believed to be connected with its multidrug tolerance (Muranaka et al. 2012). Zinc (Zn), similar to Cu, is toxic to cells at high concentrations, reducing both planktonic and biofilm cells growth. However, it could promote EPS production and cell-to-cell

adhesion. Biofilm cells accumulates more Zn than planktonic cells (Cobine et al. 2013; Navarrete and De La Fuente 2014). Studies carried by our group showed that the *X. fastidiosa* knockout mutants of Zn homeostasis (*zur*) and Zn detoxification (*czcD*) genes can significantly reduce foliar symptoms and bacterial populations. Detoxification of Zn is crucial for *X. fastidiosa* full virulence in planta (Navarrete and De La Fuente 2015). Iron (Fe) influences *X. fastidiosa* growth and siderophores production (Silva-Stenico et al. 2005). Fe-induced genes are related with function of type I and type IV pili, which are necessary for *X. fastidiosa* full virulence (Zaini et al. 2008). Calcium (Ca) in *X. fastidiosa* affects key virulence traits of *X. fastidiosa* including twitching motility, biofilm formation, cell adhesion and aggregation, extracellular compounds production and others (Cruz et al. 2012; Cruz et al. 2014; Parker et al. 2016).

Ionome modification occurs in host plants when they are infected by pathogens (De La Fuente et al. 2013). Our group compared the ionome analysis of *X. fastidiosa*-infected host plants and non-infected host plants. The results from both greenhouse (tobacco) and field samples (grapevine, blueberry and pecan) indicate that the infection of *X. fastidiosa* causes plant ionome changes (De La Fuente et al. 2013). These changes correlated with virulence of *X. fastidiosa* isolates (Oliver et al. 2014). Infection of *X. fastidiosa* could lead to a non-significant increase of Cu in host plant leaves (De La Fuente et al. 2013). This change was significant when plants were amended with additional Cu, and the increased Cu inside plants tend to cause severe foliar symptoms (Ge et al. 2020). Infection with *X. fastidiosa* causes a significant increase of leaf Ca content and decrease of leaf P. Other elements, such as Fe, Zn, Mg, Mn and others, are non-significantly changed in *X. fastidiosa* infected leaves. Mechanisms of these changes are still unknown, but it was hypothesized that could be correlated with plant defense strategies (De La Fuente et al. 2013;

Navarrete and De La Fuente 2015; Oliver et al. 2014). The ionome changes of *X. fastidiosa* and host plants during the infection process are of interest. A better understanding of plant and pathogen interaction could be achieved by studying the ionome changes during infection.

Copper Used as Bactericide and Resistance Acquisition

Cu is an essential element that is required for metabolic activity in bacteria. It serves as an electron donor and acceptor in the cell by the redox reactions between Cu(I) and Cu(II). This makes Cu important for bacterial cells for metalloprotein biosynthesis and redox reaction catalyzation (Brown et al. 1992). However, the intracellular chemical reactions of Cu also makes it toxic, as the structure and function of protein, lipid and nucleic acids change with high intracellular Cu (Brown et al. 1992; Sharma and Dietz 2009; Solioz et al. 2010). Therefore, Cu-based compounds have been used as chemical control for many plant diseases in agriculture (Rusjan 2012).

The first record of using Cu-based compounds in agriculture goes back to 1761, when copper sulphate ($CuSO_4$) was discovered as having antibacterial effects and used on seeds (Rusjan 2012). Later in the 1880s, a mixture of $CuSO_4$ and lime in water, called Bordeaux mixture, started to be widely used to control fungal disease, especially for downy mildew of grapevine (Brown et al. 1992; Brun et al. 1998). At that time, Bordeaux mixture and its modified compound Burgundy mixtures turn out to be indispensable fungicides to control various diseases of plants. Later, more Cu-based microbicides were discovered, and the common form of them are sulphates, oxychlorides, acetates, carbonates, oleates, silicates, hydroxides, etc., which are

soluble. Heretofore, many thousands of tons of Cu are used annually in agriculture all over the globe (Besnard et al. 2001; Parat et al. 2002; Provenzano et al. 2010).

Cu-based bactericides are wildly used due to non-curative or non-systemic activity (Behlau 2010). These compounds can act against bacteria based on their ability to be retained on plant surfaces, reducing inoculum build-up on susceptible leaf flushes. Moreover, they can also protect expanding fruit surfaces from infections, which could save the fruit from early-infection-caused fruit drop (Behlau 2010; McGuire 1988). Although disease such as citrus canker can be reduced by Cu-based bactericides, the inoculum in orchard and field could not completely be destroyed by Cu-based bactericides. Therefore, to minimize crop losses, growers repeat Cu spray applications season after season and year after year (Bender and Cooksey 1987; Graham et al. 2010). As a consequence of the human activities, Cu and Cu-contained compounds are widespread in the environment. Among these activities, the priority is Cu-based compounds applied as bactericides and fungicides in agriculture (Rusjan 2012). The Cu concentration in many fields is already high enough to attract people's attention. In the published literature, Cu concentrations in vineyard surface soil in the European Union is often exceeding 200 mg/kg, the acceptable threshold of Cu in agricultural soil (Brunetto et al. 2016). Cu content in soil of vineyards are many times higher than that of forests in same region (Rusjan et al. 2007). Exceptionally high concentrations of Cu in vineyard soil have been observed in France (above 1000 mg /kg) and in Brazil (above 3000 mg /kg) (Kunito et al. 1999; Mirlean et al. 2007).

The over exposure to Cu-enriched environments enable microbes to build up well-developed Cu resistance strategies. Several studies have pointed out a correspondence between Cu contamination and the frequency of Cu tolerant/resistant bacterial isolates (Brun et al. 2001; Kunito et al. 1999). The wide use of Cu in agriculture sets a selective pressure for plant

pathogens. This makes it crucial for pathogens to come up with strategies for surviving under environmental Cu stress. Once pathogens acquired Cu resistance, the frequency of the resistant population among them will gradually increase under the selective pressure of continuous applications of Cu-based bactericides (Behlau et al. 2011). Different studies have shown the identification of Cu resistant strains among many plant-pathogenic bacterial species, such as *Pseudomonas* spp. (Andersen et al. 1991; Gutiérrez-Barranquero et al. 2013), *Pantoea* spp. (Nischwitz et al. 2007), *Erwinia* spp. (Al-Daoude et al. 2009) and *Xanthomonas* spp. (Behlau et al. 2011; Lee et al. 1994). The high frequency of Cu resistance reflects the interactions between environment and bacteria. Gene adaptation as well as the horizontal gene transfer of resistance genes play a key role in this process (Berg et al. 2005). For example, the study of sequence alignments of *copLAB* (cop: copper resistance) genes from different strains or species of *Xanthomonas* suggested that resistance genes are highly conserved (identity of nucleotide sequences >90%) among Cu resistant strains (Behlau et al. 2013). This high frequency of conservation among genes of these strains suggests a common origin of Cu resistance in Xanthomonads. While phylogenetic analysis also showed there are slight differences in nucleotide sequences within groups of strains. This demonstrates that Cu resistance genes could also been independently exchanged among different species of Xanthomonads throughout the world (Behlau et al. 2011).

Cu Resistance *cop* Gene Family

The *cop* (copper resistance) gene family is a well-known Cu resistance family in many plant pathogenic bacteria, with homology to the *pco* (plasmid-borne copper resistance) gene family in

E. coli. In *Pseudomonas syringae* the *cop* gene family includes six members (*copABCDRS*), which are encoded in a plasmid. For example, in *P. syringae* pv. *tomato* the *copABCD* operon is encoded in pT23D (Mellano and Cooksey 1988a, b). The expression of the operon is regulated by a two-component system *copRS* (Brown et al. 1995; Mills et al. 1993). *cop* gene family and its homologous genes are also found in *Xanthomonas* spp. Although not all Cu resistant strains have *copCDGMF* genes, *copLAB* and their homologous genes are present in almost all Cu resistant strains of *Xanthomonas* spp (Behlau et al. 2011). Study of Cu resistance strains of *Xanthomonas citri* subsp. *citri* and *X. alfalfae* subsp. *citrumelonis* showed that *copL, copA* and *copB* are the main genes responsible for their resistance (Behlau et al. 2011).

Using DNA probes, a study showed that *cop* genes have close homologous genes in many plant pathogenic bacteria. These homologues are present in Cu resistance strains of *P. cicchorii, P. putida* and *P. fluorescens* (Cooksey et al. 1990). Part of the *cop* operon has a chromosomal homologue. In both plasmid and chromosomal DNA from the *P. cicchorii* and *P. fluorescens* strains, homologous genes were detected. In addition, homologues were also detected in several nonresistant strains (Cooksey et al. 1990). In *Agrobacterium tumefaciens,* which is a soil-borne plant-pathogenic bacterium causing crown gall tumors worldwide, the Cu resistance determinants *copARZ* are located on its circular chromosome (Stoyanov et al. 2001). The above-mentioned cases and some other cases in *Pseudomonas* (Lim and Cooksey 1993) and *Xanthomonas* (Basim et al. 2005; Lee et al. 1994), showed that Cu resistance determinants can also be located in the chromosome. But in plant pathogenic bacteria Cu resistance determinants are located predominantly in plasmids (Rusjan 2012). This characteristic is believed to facilitate movement of Cu resistant genes among different bacterial species and transfer to Cu sensitive strains (Rusjan 2012).

The *copA* gene is a key gene in the *cop* operon and is present in almost all plant pathogenic bacteria that have *cop* gene family. However, its cellular localization and protein function varies among different bacteria. In *P. syringae* (Ps), the *copA* gene encodes a periplasmic protein (72KDa). PsCopA protein was abundant when this bacterium was induced with $CuSO_4$, accounting for 3% of total cellular protein (Cha and Cooksey 1991). Based on the experiment results of the Cha and Cooksey (1991) group, PsCopA has 11 Cu atom binding sites. One is type I Cu binding site (His-X_{48}-Cys-X_4-His-X_4-Met), which is usually found in multi-Cu oxidase enzymes, located at its C-terminus. There are multiple repeats of the motif Asp-His-X_2-Met-X_2-Met in PsCopA sequence which is highly possible that they serve as binding sites for Cu, supported by site-specific mutagenesis experiments and physical bio-chemical analyses (Cha and Cooksey 1991; Cooksey 1993). In the *X. citri* pv. *citri* (Xc) genome, homologues of the *PscopA* genes were identified. *XccopA* is sequentially located on the *X. citri* pv. *citri* chromosome and is transcribed as an unique transcript (Behlau et al. 2011; Teixeira et al. 2008). The results of cellular localization showed that XcCopA protein was detected only in the cell cytosol (Teixeira et al. 2008). Analysis of CopA protein sequence alignment from *Xanthomonas* species reveals it has two highly conserved domains: one is extending from the N-terminal region to the middle of the protein, and another is located at the C-terminal region. The conserved domain at the C-terminus contains the putative type I Cu-binding site (His-X_{48}-Cys-X_4-His-X_4-Met) (Teixeira et al. 2008). On the other hand in *A. tumefaciens*, *copA* encodes a putative protein (90.1kDa) that is a CPx-type ATPase functioning as a transmembrane Cu efflux pump. Its amino acid sequence shares high levels of identity to other bacterial Cu ATPases (Nawapan et al. 2009).

The *copB* gene is usually located next to the *copA* gene in the same operon, and shares an intergenic region with the *copA* gene in some cases (Teixeira et al. 2008). In *P. syringae*, *PscopB*

gene encodes an outer membrane protein (39KDa). The prediction of PsCopB protein showed that it has 5 repeat sequences (Asp-His-X_2-Met-X_2-Met). Although there is no direct evidence proving Cu-binding activity of PsCopB protein, it is tempting to speculate that repeat sequences are involved in binding Cu (Cooksey 1993; Ouzounis and Sander 1991). In *X. citri* pv. *citri*, *XccopB* gene is located in the chromosome and encodes a cytoplasmic membrane protein (Behlau et al. 2011; Teixeira et al. 2008). In *X. fastidiosa* subsp. *pauca*, the expression of the *copB* gene is significantly upregulated under 5mM $CuSO_4$ in biofilm cells (Rodrigues et al. 2008), but its cellular location has not been determined.

The *copC* gene of *P. syringae* encodes a periplasmic protein (72KDa), which binds only one Cu atom per polypeptide. Similar to PsCopA, the PsCopC protein is abundant in cells, accounting for 1% of the total cellular protein, when cells were induced by $CuSO_4$ treatments (Cooksey 1993). The Cu binding site of PsCopC is the repeat sequence (Asp-His-X_2-Met-X_2-Met) as in PsCopB and PsCopA. PsCopD has extensive internal regions of hydrophobic amino acids (McGuire 1988). The PsCopA, PsCopB and PsCopC have leader sequences and PsCopD has extensive internal regions of hydrophobicity (Mellano and Cooksey 1988a, b), suggesting membrane localization. In the *X. citri* pv. *citri* genome, homologues of the *copC* and *copD* genes from the *cop* operon are absent. In *X. campestris* pv. *juglandis* ORFs 3 and 4 are identified. They are 47% and 40% identity with *copC* and *copD* from *P. syringae* pv. *tomato,* respectively (Lee et al. 1994).

Function of *cop* Family

As the first plant pathogen that its Cu resistance been characterized at the molecular level, *P. syringae* pv. *tomato* has a better defined function of *cop* family compared with other plant pathogens (Bender and Cooksey 1987). The functional descriptions of the Cop proteins in *P. syringae* provide great references for studying *cop* genes-related Cu resistance in gram-negative plant pathogenic bacteria. The experiments carried by Cooksey's group showed that when *P. syringae* was grown on Cu-containing media, colonies turn to blue color, due to the increase of Cu accumulation in cell. Moreover, when Cu-resistant *P. syringae* strain PT23 was grown on Cu-containing media, transmission electron micrographs showed that Cu was accumulated around the perimeter of the cell (Cooksey 1993). PsCop proteins were proposed to play the role of Cu accumulation in the cell periplasm and outer membrane. The over expression of *P. syringae* pv. *tomato cop* genes *in vivo* was helpful to study their function (Bondarczuk and Piotrowska-Seget 2013; Puig et al. 2002). Subcellular localization, Cu binding capacities and amino acid sequences of the proteins suggest a mechanism in which the *cop* determinants confer resistance to Cu by sequestration of Cu ions outside the cell (Brown et al. 1992). Cu is sequestered by the periplasmic proteins PsCopA and PsCopC and probably by the outer membrane protein PsCopB. For PsCopC and PsCopD, mutational analysis indicates they are both responsible for Cu accumulation in the cytoplasm. PsCopC may deliver Cu to PsCopD, which transports Cu through the inner membrane into the cytoplasm (Arnesano et al. 2002; Cooksey 1993). However, some experimental results suggest that PsCopC and PsCopD, which are probable inner membrane proteins, function in Cu uptake (Cha and Cooksey 1993). J, S, Cha and D. A. Cooksey expressed these two genes in a *P. syringae* strain lacking the plasmid containing these genes. When *PscopC* and *PscopD* were expressed without *PscopA* and *PscopB* functioning, the bacterium hyposensitized Cu. While, when they expressed PsCopC and PsCopD

with *PscopA* together, hypersensitivity was not observed (Cha and Cooksey 1993). Thus, researchers concluded that on the one hand *cop* family regulates Cu sequestering activity that happened in the periplasm and outer membrane of cell. On the other hand, the supply of Cu, provided by some compensating transport activity, is regulated by the *cop* family (Cha and Cooksey 1993; Cooksey 1993). In *X. citri* pv. *citri*, the mutant strains of the *copAB* operon are extremely sensitive to Cu compared with the wild type strain. This indicates the *copAB* operon is crucial for its Cu resistance (Teixeira et al. 2008)

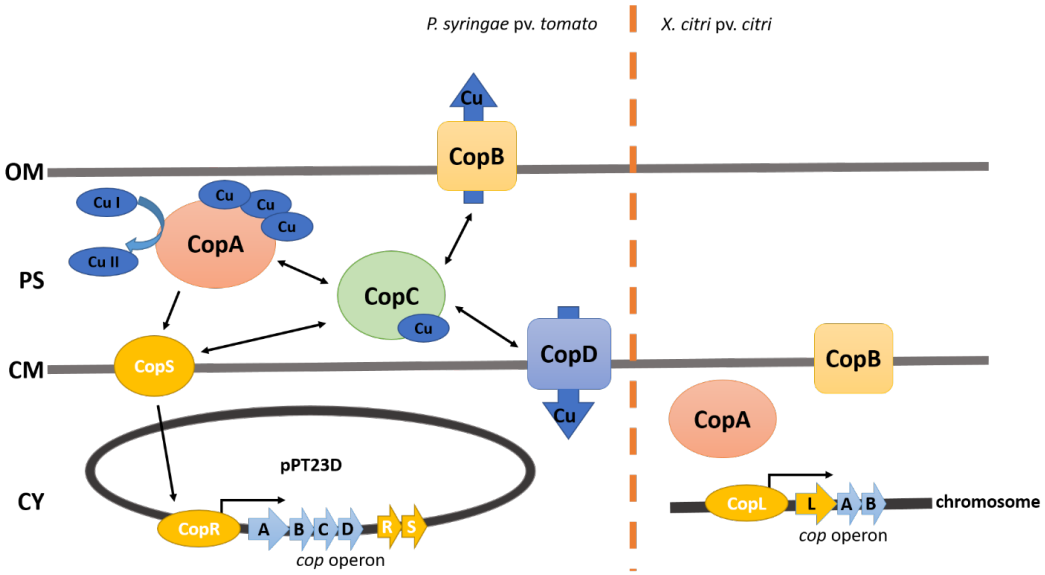

Fig. 1 Models for *cop* system in *P. syringae* pv. *tomato* and *X. citri* pv. *citri*

Abbreviations: OM: outer membrane, PS: periplasmic space, CM: cytoplasmic membrane, CY: cytoplasm. Arrows indicate presumed interactions between proteins (model of *P. syringae* pv. *tomato* modified from Puig et al. 2002 and Bondarczuk and Piotrowska-Seget 2013. model of *X. citri* pv. *citri* drew based on Teixeira et al. 2008)

Regulation of *cop* Family

Experiments have shown that the *cop* genes expression is induced specifically by Cu. The known regulatory systems of the *cop* family are *copRS* in *Pseudomonas* spp. and *copL* in *Xanthomonas* spp. In *P. syringae*, the plasmid and chromosomal regulatory genes interact with each other and are together involved in regulation of Cu resistance. It is proposed that the regulation of *cop* genes in *P. syringae* is negative based on the results of mobility-shift experiments with a *cop* promoter contained DNA fragment (Mills et al. 1994). When Cu is absent, a repressor encoded in the chromosome binds to the promoter and prevents the expression of the *cop* operon. When Cu is present, the repressor releases from the promoter and the *cop* genes are expressed (Mills et al. 1994). The repressor release process needs two genes *PscopR* and *PscopS*, which are plasmid-borne and adjacent to the *cop* operon. PsCopS and PsCopR form a two-component regulatory module that is constitutively transcribed. PsCopR binds at the 35 region of the *cop* promoter to a conserved motif (*cop* box) in *P. syringae* (Mills et al. 1993; Mills et al. 1994). PsCopS is predicted to be a Cu sensor located at the inner membrane that presumably interacts with *PscopA* or *PscopC* (Puig et al. 2002). The studies of the Cu resistance in *Xanthomonas* spp. indicate that the most likely mechanism of Cu resistance is through cellular Cu sequestration and accumulation activity. Instead of *copRS* regulation system as described in *Pseudomonas*, *copL* regulates the expression of *copA* and *copB* in *Xanthomonas* spp. (Basim et al. 2005; Cooksey et al. 1990; Stall et al. 1986). In *A. tumefaciens* (At), CopR (15.55 kDa) is a transcriptional regulator of *cop* operon. For regulation, it is suggested that AtCopR binds to the *AtcopARZ* promoter and represses the expression of the *cop* operon under Cu-limited conditions. By this regulation, bacterial cells can maintain their essential Cu ion needs. When Cu concentration was increased, AtCopR is activated and forms a transcriptional activator, thereby enhancing

AtcopARZ transcription. This efficient mechanism allows *A. tumefaciens* to survive in high Cu environments (Nawapan et al. 2009).

Cu Tolerance *cut* Family

The *cut* (Cu tolerance) gene family is believed to be related with Cu tolerance in different organisms. The *cut* gene family of *E. coli* had six members: *cutABCDEF*. They are involved in Cu uptake, storage, delivery and efflux. Mutation of one or more *cut* genes leads to increased Cu sensitivity (Gupta et al. 1995; Rouch et al. 1989). The *cutA* gene is located on the chromosome of *E. coli* and it contains two operons and encodes 3 proteins CutA1 (13 kDa), CutA2 (50 kDa) and CutA3 (24 kDa). CutA1 is a cytoplasmic protein, while CutA2 and CutA3 are inner membrane proteins (Fong et al. 1995). The *cutA* gene was originally proposed to encode a cellular Cu transporter (Rouch et al. 1989). Experiments showed that CutA was not an exclusive transporter of Cu, since it may also be related with Co^{2+}, Ni^{2+} and Zn^{2+} regulation in cells (Fong et al. 1995). CutA1 and CutA2 proteins are involved with Cu tolerance, which was proven by the study of their mutations, while, the mutant of *cutA3* did not show any distinguished phenotype (Fong et al. 1995). *cutE* is the first *cut* gene that has been sequenced and analyzed, and is located in the chromosome encoding a 56 kDa protein. At the beginning, CutE was proposed to be a soluble protein and play a role in Cu sequestration (Rogers et al. 1991). Later, it was believed to be located in the inner cellular membrane and serve as an inner membrane lipoprotein transacylase (Fong et al. 1995; Gupta et al. 1993). CutF is an outer membrane protein with the motif (M-X-X-M-X-X-X-M), which is similar to the conserved metal-binding domains in type I Cu proteins. CutF has potential Cu-binding ability (Gupta et al. 1995).

Rouch et al. (1989) proposed that CutC encodes an efflux protein functioning as removing excess Cu from the cytoplasm. Later, Gupta et al. (1995) carried deduced amino acid sequence analysis of CutC protein showing that it is a hydrophilic protein, it has multiple charged residues with a possible Cu-binding motif (M-X-X-M-X-X-X-M) at the N terminal. Experiments showed mutation of the *cutC* gene lead to intracellular accumulation of Cu without changing Cu uptake kinetics. Therefore, CutC is suggested to be a cytoplasmic Cu-binding protein (Gupta et al. 1995). The CutC protein has been proved to be required for Cu tolerance in *E. coli*. It is possible that CutC serves as a cytosolic component of an efflux pathway for Cu. The increase of Cu sensitivity of its mutant is due to a decrease of Cu efflux (Gupta et al. 1995; Rouch et al. 1989). The expression of the *cutC* gene is significantly upregulated under 5mM $CuSO_4$ in biofilm cells of *X. fastidiosa* subsp. *pauca* (Rodrigues et al. 2008). However, the *cutC* gene is downregulated in the nematode *Caenorhabditis elegans* under increased Cu concentration (Calafato et al. 2008). In *Enterococcus faecalis* (Ef), *cutC* gene is induced at late time points under Cu exposure. EfCutC may function as a secondary response after the early transcriptional response carried by *cop* genes. *EfCutC* mutant shows increased intracellular Cu accumulation. It is suggested that EfCutC functions as a chaperone or a Cu-usher protein in the control of Cu homeostasis and Cu efflux through directly or indirectly mechanisms (Latorre et al. 2011). CutC, which is conserved among prokaryotic and eukaryotic organisms, is also studied in animals and human beings in regard to protein structural features and physiological function (Gupta et al. 1995; Li et al. 2010). The human homologue of *cutC* (*hCutC*) encodes a 30 kDa cytoplasmic protein (Ota et al. 2004), and it was found expressed in nearly all human tissues (Li et al. 2005). The analysis of biochemical and structural characteristics of hCutC protein reveal that it has a typical triosephosphate isomerase (TIM)-barrel fold and three conserved residues, one is on the inner

surface of the C-terminal end of the TIM-barrel, and the other two are strictly conserved residues Cys31 and Cys52. These conserved residues could be potential Cu(I) binding sites. Some researchers believe that hCutC does not function as a Cu(I) transporter, but as an enzyme with Cu(I) as a cofactor (Li et al. 2010). Silencing of *hCutC* in HepG2 cells did not change total cellular Cu, however, it leads to Cu sensitivity. It is possible that hCutC did not participate in cellular Cu export. The imbalanced distribution and metabolism of intracellular Cu could be the reason of induced Cu sensitivity. Interestingly, the onset of apoptosis was noticed in *hCutC*-silenced cells by morphological analysis. The decreased mitochondrial ΔΨm, noticed in their results, shows that *hCutC*-silenced cells are under a Cu mediated metal stress and leads to apoptosis (Kunjunni et al. 2016). The putative functions of the *cutC* gene are various among different organisms, while it seems to be always crucial for Cu tolerance. The study of the *cutC* gene in the plant pathogen *X. fastidiosa* is important for unveiling its Cu resistance/tolerance mechanisms, and fulfillment of the knowledge of Cu resistance/tolerance in plant and human pathogens.

Research Goals

X. fastidiosa as a plant pathogenic bacterium causes worldwide diseases in many plants and draws attention as significant economic losses are caused by this pathogen. One of the main environments where this bacterium is found are vineyards, where usually Cu accumulates to a high amount in the soil as a consequence of yearly usage of Cu-based antimicrobial compounds. How *X. fastidiosa* handles and reacts to extra Cu in the environment is an area of interest.

However, there is little information on this topic. Meanwhile, the knowledge of *copA*, *copB* and *cutC* genes, which have been proved to be related with Cu homeostasis/resistance in many bacteria, were almost uncharacterized in *X. fastidiosa*. Whether these genes would function as their homologues in other bacteria is unclear. To fill the gaps in knowledge and illuminate the mechanism of Cu homeostasis/resistance in *X. fastidiosa,* I developed my dissertation. My research will help to better understand the mechanisms of Cu-related virulence changes in the plant pathogen *X. fastidiosa*, to ultimately achieve the goal of disease control and management in the field.

In this study, we investigated the influence of Cu on the plant pathogen *X. fastidiosa* and its virulence, to understand whether Cu amendments in plants would help disease symptom control. Meanwhile, I performed research to understand Cu homeostasis/resistance in this pathogen, by functional analysis of three Cu related genes (*copA*, *copB* and *cutC*) in vitro and in planta. Last but not the least, what features of *X. fastidiosa* will influence its Cu resistance ability are discussed in the last chapter of this dissertation.

Research Objectives

1) Determine the effect of $CuSO_4$ on control of *X. fastidiosa*-caused diseases: In this objective, I want to understand if $CuSO_4$ uptake by plants is effective to inhibit *X. fastidiosa* colonization in xylem vessels, and will it have an effect on disease development? We hypothesize that Cu added to plants will control *X. fastidiosa* growth inside xylem vessels.

2) Characterize *copA* and *copB* genes function in *X. fastidiosa*: In this objective, I conducted mutagenesis of the target genes and functional analysis of virulence traits, including planktonic and biofilm growth, resistance to Cu and virulence in plant, and determined if *copA* and *copB* are important for Cu homeostasis/resistance in *X. fastidiosa*.

3) Characterize *cutC* gene function in *X. fastidiosa*: In this objective, I assessed if *cutC* is important for Cu homeostasis/resistance in *X. fastidiosa*. We hypothesized that *X. fastidiosa*, which infects plants under high soil Cu content, has evolved efficient Cu homeostasis/resistance mechanisms.

4) Characterize features that influence Cu homeostasis/resistance of *X. fastidiosa*: In this objective, I intend to analyze the phylogeny of Cu-related genes in *X. fastidiosa* and if the ability of Cu homeostasis/resistance is correlated with location and host of isolation.

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Chapter 2: Copper supplementation in watering solution reaches the xylem but does not protect tobacco plants against *Xylella fastidiosa* infection

Abstract

Xylella fastidiosa is a xylem-limited plant pathogenic bacterium that causes disease in many crops worldwide. Copper (Cu) is an antimicrobial agent widely used on *X. fastidiosa* hosts to control other diseases. While the effects of Cu for control of foliar pathogens is well known, it is less studied on xylem-colonizing pathogens. Previous results from our group showed that low concentrations of $CuSO_4$ increased biofilm formation, while high concentrations inhibited biofilm formation and growth in vitro. In this study, we conducted in planta experiments to determine the influence of Cu in *X. fastidiosa* infection using tobacco as a model. *X. fastidiosa*-infected and non-infected plants were watered with tap water, or water supplemented with 4 mM or 8 mM $CuSO_4$. Symptom progression was assessed, and sap and leaf ionome analysis was performed by inductively coupled plasma-optical emission spectroscopy (ICP-OES). Uptake of Cu was confirmed by increased concentrations of Cu in sap of plants treated with $CuSO_4$-amended water. Leaf scorch symptoms in Cu-supplemented plants showed a trend towards more severe at later time points. Quantification of total and viable *X. fastidiosa* in planta indicated that $CuSO_4$-amended treatments do not inhibit but slightly increased the growth of *X. fastidiosa*. Cu in sap was in the range of concentrations that promote *X. fastidiosa* biofilm formation according to our previous in vitro study. Based on these results, we proposed that the plant Cu homeostasis machinery controls the level of Cu in xylem, preventing it from becoming elevated to a level that would lead to bacterial inhibition.

Introduction

Xylella fastidiosa is a gram negative plant-pathogenic bacterium that causes diseases in many economically important crops worldwide, such as grape, citrus, plum, coffee, blueberry, peach (Hong et al. 2015; Hopkins and Purcell 2002; Purcell and Hopkins 1996), and recently olives (Saponari et al. 2019). In the U. S., *X. fastidiosa*-caused diseases with the most economic impact are Pierce's disease (PD) of grapevine and bacterial leaf scorch (BLS) of blueberry (Oliver et al. 2015). *X. fastidiosa* is transmitted by xylem-feeding insects such as sharpshooters and leafhoppers (Janse and Obradovic 2010). Once the bacteria gets inside the plant xylem, they colonize xylem vessels and form biofilm that block water and nutrients transport (Chatterjee et al. 2008; Newman et al. 2003). This blockage is thought to lead to the development of leaf scorch, which is the typical symptom of *X. fastidiosa* (Chatterjee et al. 2008). Because of the internal niche (xylem) that *X. fastidiosa* colonizes, regular application methods of antibacterial treatments such as foliar sprays cannot easily reach the pathogen. Therefore, controlling *X. fastidiosa*-caused disease is a significant challenge.

Cu-based microbicides have been widely used in agriculture since 1880's to control fungal diseases such as downy mildew in grape (Brun et al. 1998; Rusjan 2012) as well as bacterial diseases (Lamichhane et al. 2018). Cu is toxic to bacteria due to the disruption of essential iron sulfur cluster proteins, and production of reactive oxygen species that leads to lipid and protein damage and can trigger plant defense response (Macomber and Imlay 2009; Sharma and Dietz 2009; Solioz et al. 2010). Although diseases can be managed this way, the lack of curative or systemic activity leads to Cu spray applications year after year (Bender and Cooksey 1987;

Graham et al. 2010). Therefore, thousands of tons of Cu are used annually in agriculture all over the globe, leading to Cu accumulation in soil. Considering arable land in Europe, the highest Cu concentrations is found in vineyards (Ballabio et al. 2018), where it often exceeds the maximum residue level allowed of 200 mg/kg of Cu (Brunetto et al. 2016; Komarek et al. 2010). Exceptionally high concentrations of Cu in vineyard soils have been observed in France (> 1000 mg/kg) (Flores-Vélez et al. 1996) and Brazil (> 3000 mg/kg) (Mirlean et al. 2007). As *X. fastidiosa* lives inside grapes, the bacterium could be exposed to high Cu concentrations taken from the soil by the host plant roots and distributed via the xylem. Moreover, bacteria could theoretically acquire Cu resistance from this high Cu-containing environment, such as Cu-resistance acquisition described for citrus-associated Xanthomonads (Behlau et al. 2013).

Recent research from Italy reported that a Zn and Cu-containing citric acid biocomplex fertilizer (Dentamet®) could influence *X. fastidiosa*-infected olive trees with changes in the polyphenolic and sugars content patterns, which may help plant defense against this pathogen infection (Girelli et al. 2017). The authors concluded that the Cu-Zn accumulation using this complex reduced the progression of symptoms caused by *X. fastidiosa* in olive trees (Girelli et al. 2017; Scortichini et al. 2019). Previous results from our group showed that Cu influences *X. fastidiosa* growth in vitro. Addition of low Cu concentrations ($CuSO_4$ 5-200 µM) to growth media increased biofilm formation in vitro, while high Cu concentrations ($CuSO_4$ > 200µM) reduced cell growth and therefore prevented biofilm formation. Additionally, Cu in growth media influenced other virulence traits such as cell-cell adhesion (Cobine et al. 2013). But the question of Cu treatments on growth of *X. fastidiosa* in planta is still unclear. In this study, we aimed to understand if $CuSO_4$ uptake by plants is effective to inhibit *X. fastidiosa* colonization in xylem vessels, and if its accumulation has an effect on disease development.

Materials and Methods

Bacterial strains and culture conditions

Xylella fastidiosa strain WM1-1 (Oliver et al. 2014; Parker et al. 2012) was used in this research, and was grown using Periwinkle Wilt (PW) agar plates (Davis et al. 1981) or Pierce's Disease 2 (PD2) broth (Davis et al. 1980) incubated at 28℃. Cell suspensions of *X. fastidiosa* used for plant inoculation (see below) were prepared following a previously-described protocol with some modifications (De La Fuente et al. 2013). Briefly, *X. fastidiosa* strain WM1-1 was streaked from -80 °C frozen glycerol (20%) stocks on PW agar plates for 5-7 days, and then re-streaked onto new plates for additional 5-7 days. Next, bacterial cultures from PW agar plates were scraped and suspended in PBS (phosphate-buffered saline). Finally, bacterial suspensions were diluted to an optical density (OD_{600}) equal to 0.8 and used for plant inoculation.

Greenhouse experiments

Tobacco plants (*Nicotiana tabacum* 'Petite Havana SR1', plant introduction number 552516) were used as a model to study *X. fastidiosa* virulence and its host interaction, as previously described (De La Fuente et al. 2013). Tobacco seeds were obtained from the United States Department of Agriculture Germplasm Resources Information Network. Seeds were germinated in Sunshine® Mix #8 (Sun Gro Horticulture Canada Ltd.). After ~3 weeks, seedlings were

transplanted to 4.5-in round pots. All plants were grown in the greenhouse at 20 to 25℃ with natural sunlight. Plants were fertilized when symptoms of deficiency were shown (yellowing of leaves). Osmocote (Outdoor & Indoor Smart-Release Plant Food; The Scotts Company, Marysville, OH, U.S.A.), a slow-release fertilizer was added three times during the growing season. After additional ~3 weeks of growth, plants were inoculated following a previously described method (De La Fuente et al. 2013; Francis et al. 2007). Briefly, only the three healthy lower leaves were kept, while upper leaves were trimmed and discarded. Two mL (OD_{600}=0.8) of the bacterial inoculum was prepared as described above. A 23-gauge needle was used to puncture small wounds in the base of the leaf petioles that were quickly covered with 20 µl of inoculum that was absorbed by the plant. Twenty-four plants were inoculated with WM1-1 strain and other 24 plants were injected with PBS buffer only (non-inoculated control). A second inoculation was performed one week after the first one following the same method. Two weeks after cutting, new shoots grew from the trimmed leaf positions; therefore trimming was performed to keep only one shoot for later growth. Cu-amended plant treatments were carried out by watering each pot either with 200 mL 4 mM $CuSO_4$, 8 mM $CuSO_4$ or with tap water (control). These Cu treatments were added once per week. The rest of the time all plants were watered with the same amount of tap water. Treatments with additional Cu started one day after the first pathogen inoculation. At ~80 days after the first inoculation, symptoms (leaf scorch) started to show. The number of plants showing leaf scorch, and the number of leaves showing leaf scorch symptoms per plant were recorded weekly and used as measurements of disease incidence and severity, respectively. Disease severity was used to calculate the area under the disease progress curve (AUDPC). AUDPC was calculated based on the midpoint rule method (Campbell and Madden 1990) as follows: $AUDPC= \Sigma [(y_i + y_{i+1})/2](t_{i+1} – t_i)$, where i = the

number of assessment times, y = disease severity score for each plant at each assessment, and t = time at each assessment. At the end of symptom development (around 4 weeks after first symptoms were observed) samples were collected for further analysis as described below. An independent experiment was performed to determine Cu phytotoxicity in tobacco plants. Eight plants for each treatment were watered with 200 mL of either 12 mM $CuSO_4$, 16 mM $CuSO_4$ or tap water (control) once per week. The rest of the time, all plants were watered with the same amount of tap water. Symptom of phytotoxicity were recorded over time.

Ionome Characterization

Five leaves above the inoculation point from each one of the five selected plants from each treatment were collected for ionome analysis, following a previously-described protocol with some modifications (De La Fuente et al. 2013). Briefly, whole leaves were dried immediately after collection by incubation at 65°C for 1 hour. These leaves were then ground to a fine powder with a mortar and pestle. For each sample, 10 mg of the powder was taken and digested in 200 µl of mineral-free concentrated nitric acid (OPTIMA, Fisher Scientific) at 100°C for one hour. After digestion, samples were diluted with 800 µL mineral-free water, followed by centrifugation at 13,000x g to remove remaining particulates. Finally, samples were analyzed by Inductively Coupled Plasma with Optical Emission Spectroscopy (ICP-OES, Perkin Elmer 7100 DV, Waltham, MA) with simultaneous measurement of Ca, Fe, Cu, Zn, Mn, S, Mg, K, Na, and P. Mineral elements concentrations were determined by comparing emission intensities to standard curves created from certified standards (SPEXCertiprep, Metuchen, NJ). Standard curves were confirmed by reanalysis of standard solutions diluted in a matrix equivalent to the

sample. Individual readings (average of two intensity measurements) and repeated analysis of individual samples, showed less than 5% variation (data not shown).

For sap ionome analysis, 5 plants from each treatment group were selected, and one sap sample was collected from each plant. Sap samples were collected using a Scholander pump model 600 pressure chamber (PMS Instrument Company, Corvallis, OR) as described previously (Oliver et al. 2014). Samples were frozen at -20°C for storage no more than one week. Samples were diluted with mineral-free water and analyzed by ICP-OES as described above for leaf samples.

Total and viable bacterial population quantification

Bacterial population quantification was done by quantitative polymerase chain reaction (qPCR) for total bacteria following a previously-described protocol (De La Fuente et al. 2013), and PMAxx-qPCR for viable cells. According to the product description (https://us.vwr.com/store/product/8286393/pmatm-pmaxxtm-pma-litetm-and-related-products-biotium), PMAxx (Biotium, Hayward, CA, USA) is a photo-reactive, cell membrane-impermeable dye that could bind with dsDNA, which then is no longer amplified by PCR. Thus, only dsDNA from live cells that was not bound to PMAxx could be amplified by PCR. Five petioles (positions 1-5 above inoculation point, from bottom to top) from five selected plants per treatment were collected from both inoculated and non-inoculated plants. Petioles were cut into small pieces, and 100 mg samples were put into 2 ml tubes with 2.0 mm beads (ZIRCONIA, Biospec Products), and ground for 2-3 min at high speed by using a mini Beadbeater 96 (Biospec Products). Samples were re-suspended with 1.5 ml PD2 broth. From each sample 500 µl were transferred to a new tube, and stored at -20°C for later DNA extraction and quantification of total

bacterial population. A second 500-µl sample was transferred to a new tube for PMAxx treatment and quantification of viable bacterial populations. To assess the accuracy of PMAxx treatment for plant tissue samples, dead bacteria cell controls were prepared by treating ground petiole samples in a 95°C water bath for 30mins. PMAxx treatment was added to samples in the dark, mixed and incubated for 5 mins. Then samples were exposed for 10 min to a strong light (700W halogen portable work light, Husky Co., Atlanta, GA). Ice was used to prevent overheating samples from light treatment. Samples were saved at -20°C for later analysis. DNA of total and viable bacteria samples were extracted using a modified CTAB protocol (Doyle and Doyle 1987), and *X. fastidiosa* populations were quantified using qPCR and a standard curve as previously described (De La Fuente et al. 2013).

Statistical analyses

For the analysis of sap concentration of Cu, all pairwise multiple comparison procedures carried out by Tukey's test using SigmaPlot 14.0 (Systat Software, Inc. SigmaPlot for Windows) ($p < 0.050$). For the analysis of disease symptoms and AUDPC, Poisson analysis in R (3.6.1 for Windows) was used. For bacterial population quantification, Cu leaf concentration and leaf ionomes, one-way ANOVA (or Kruskal Wallis for non-normal data) was used and means separated by Fisher's protected LSD ($p < 0.05$) using Statistix 8.0 (Analytical Software, St. Paul, MN). For leaf Cu concentration, data outliers were removed by ROUT outliers test (Q = 5%) using Prism 8 for Mac OS 8.2 (GraphPad Software, CA).

Results

Copper uptake by tobacco plants

ICP-OES was used to determined Cu concentration in tobacco sap (Fig. 1) and leaves (Fig. 2). Cu concentration of plant sap in $CuSO_4$-amended treatments (4 mM and 8 mM $CuSO_4$) was significantly increased as compared to control groups (water treatment) (Fig. 1). In control groups watered with un-amended tap water, the average Cu concentration in sap was 0.68 ± 0.09 µM in non-inoculated plants and 1.00 ± 0.12 µM in *X. fastidiosa*-inoculated plants. In 4 mM $CuSO_4$-amended treatments Cu concentration was 46.12 ± 3.17 µM and 39.81 ± 4.39 µM for non-inoculated and inoculated plants, respectively. In 8 mM $CuSO_4$-amended plants Cu concentration was 103.51 ± 8.94 µM and 101.53 ± 7.15 µM for non-inoculated and inoculated plants, respectively. In independent experiments sap Cu concentrations for control groups ranged from 0.3-1.0 µM, for 4 mM $CuSO_4$-amended treatments ranged from 10-50 µM, while 8 mM $CuSO_4$-amended treatments resulted in 50-100 µM.

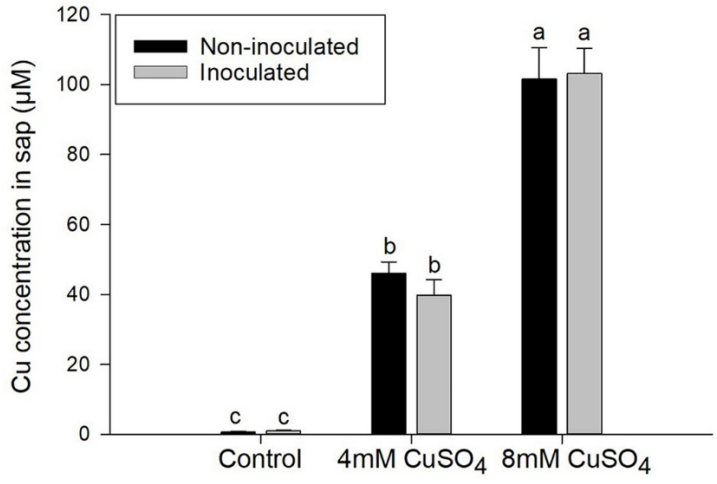

Fig. 1. Cu concentration in sap of tobacco plants

Sap samples were collected from tobacco plants (*Nicotiana tabacum* 'Petite Havana SR1') grown in the greenhouse. Half of the plants were inoculated with *X. fastidiosa* ('inoculated') and the other half inoculated with PBS ('non-inoculated'). Plants were watered either with 200mL of 4mM $CuSO_4$, 8mM $CuSO_4$ or tap water (control) (n = 5/treatment). Mean values are shown in graph, and error bars represent standard error of the mean. Data used in the graph corresponds to one representative experiment, and three independent experiments performed under the same conditions showed similar tendencies. Different letters above bars indicate significant differences ($p < 0.05$) among all treatments according to Tukey's test (all pairwise multiple comparison procedures) (SigmaPlot 14.0).

Cu concentration in plant leaves was also quantified (Fig. 2). In control groups, leaves contained 2.97 ± 0.12 mg kg^{-1} and 5.53 ± 0.41 mg kg^{-1} Cu for non-inoculated plants and inoculated plants, respectively. Leaf Cu concentration in 4 mM $CuSO_4$-amended treatments were significantly increased as compared to the control groups. For non-inoculated plants Cu concentration was 46.87 ± 5.95 mg kg^{-1} and for inoculated plants was 99.81 ± 12.67 mg kg^{-1}. In 8 mM $CuSO_4$-amended treatments Cu concentration were 60.50 ± 5.54 mg kg^{-1} and 172.52 ± 26.07 mg kg^{-1} for non-inoculated plants and inoculated plants, respectively. Leaf Cu concentrations for control groups ranged from 2-6 mg kg^{-1}, for 4 mM $CuSO_4$-amended treatments ranged from 50-100 mg kg^{-1}, while 8 mM $CuSO_4$-amended treatments resulted in 60-180 mg kg^{-1}.

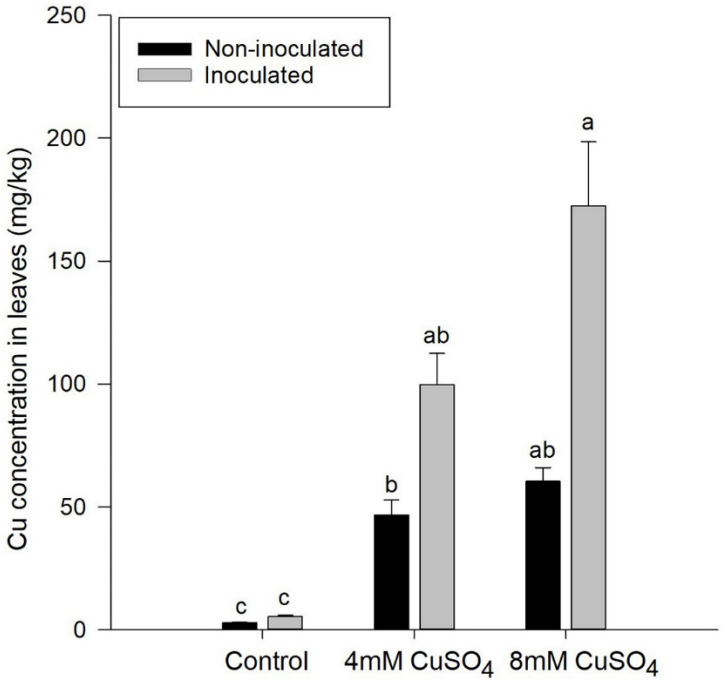

Fig. 2. Cu Concentration in leaves of tobacco plants

Leaves samples were collected from tobacco plants (*Nicotiana tabacum* 'Petite Havana SR1') grown in the greenhouse. Plants were inoculated with *X. fastidiosa* ('inoculated') or PBS ('non-inoculated'). Plants were watered with either 200ml of 4mM $CuSO_4$, 8mM $CuSO_4$ or tap water (control). Five leaves per plant and five plants per treatment were collected (n = 25/treatment). Values in graph represent means and error bars represent standard error of the mean. Data used in the graph corresponds to one representative experiment, and three independent experiments performed under the same conditions showed similar tendencies. Different letters on top of the bars indicated significant differences ($p < 0.05$), according to Kruskal-Wallis (all pairwise multiple comparison test) (Statistix 8.0).

Cu-amended treatments affect other elements in tobacco leaves

Other elements (Ca, Fe, Zn, Mn, S, Mg, K, Na and P) in leaves besides Cu were also measured by ICP-OES. As expected, increasing Cu added to plants also changed other elements in leaves

(Fig. 3). In non-inoculated plants (Fig. 3A), Fe, S, K and P were significantly decreased in Cu-amended treatments (in 4 mM $CuSO_4$ treatment). Among them, Fe concentration decreased more than 40% from the control, while P and S decreased ~60% and 40% from control, respectively. Zn, Mn and Mg concentrations increased in most cases significantly in Cu-amended treatments. The concentration of Mn in leaves showed the highest increment, ~60% more than control. In inoculated plants (Fig.3B), most of the elements show similar tendencies, but different degrees of changes as compared to non-inoculated plants. The exception were Fe and S, which show opposite tendencies. The comparison of leaf element concentration between non-inoculated and inoculated plants in control groups (water only) show the same tendencies (data not shown) as previously published (Oliver et al. 2014).

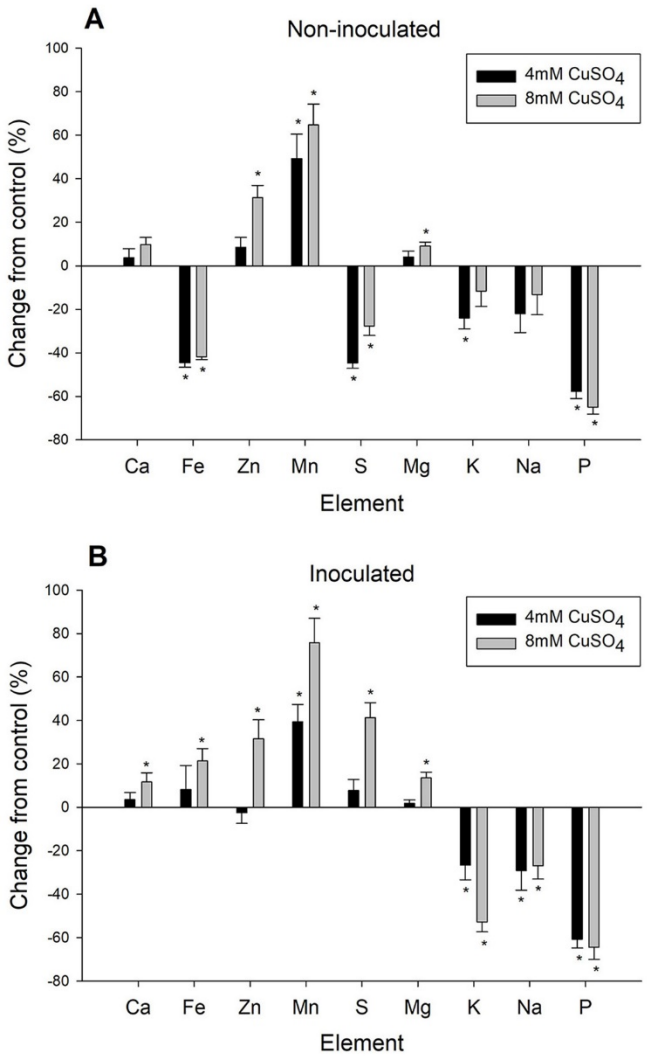

Fig. 3. Ionome changes in tobacco plant leaves under Cu-amended treatments

Ionome changes in plants inoculated with PBS ('non-inoculated') (A) or *X. fastidiosa* ('inoculated') (B). Elements Ca, Fe, Zn, Mn, S, Mg, K, Na and P were measured form the same leaves samples used for Cu concentrations (Fig. 2). Plants were watered with either 200ml of 4mM $CuSO_4$, 8mM $CuSO_4$ or tap water (control). Five leaves per plant and five plants per treatment were collected (n = 25/treatment). Values in graph represents means and error bars represent standard error of the mean. Data used in the graph corresponds to one representative experiment, and three independent experiments performed under the same conditions showed similar tendencies. *Indicates significant difference ($p < 0.05$) between treatments according to one-way ANOVA or Kruskal-Wallis.

$CuSO_4$-amended treatments had no significant effect on *X. fastidiosa* symptoms

Tobacco was shown in previous reports to be a suitable model to study *X. fastidiosa* virulence and pathogen-host interactions (De La Fuente et al. 2013; Francis et al. 2007; Oliver et al. 2014). Tobacco plants 'Petite Havana SR1' showed symptoms of leaf scorch when inoculated with *X. fastidiosa* strain WM1-1. After 75-80 dpi symptoms of leaf scorch started to show in all *X. fastidiosa*-inoculated groups (Fig. 4A). After 3 weeks post symptoms appearance (wps), all inoculated plants had similar disease progress curves. Starting at 4 wps, Cu-amended treatments showed ~60% of leaves with scorch symptoms, while the control group (water only treatment) showed ~50% leaf scorch. These differences were increased at 5 wps, when the Cu-amended treatments showed leaf scorch from 65% to 75%, while the control group showed the same symptoms at 55%. The Cu-amended treatments did not show any significant disease control effect, even showing a trend towards more severe disease than the control at later time points. After statistical analysis, only 4 mM $CuSO_4$-amended treatment had significantly higher disease symptoms than the control group ($p = 0.038$) (data not shown) at the last time point considered in one of the repetitions of the experiment. But these differences were non-significant in successive repetitions of the experiment. AUDPC of the three experimental repetitions show that symptoms of leaf scorch in the Cu-supplemented plants were generally more severe than the control but non-significantly different (Fig. 4B). These conclusions were the same in all three independent experiments.

In non-inoculated plants watered with 8 mM $CuSO_4$ minor symptoms of phytotoxicity were observed, expressed as decrease of secondary shoot growth and a general reduction in plant growth. For this reason, we established additional treatments when plants were watered with higher concentrations (12 mM and 16 mM) of $CuSO_4$, and assessed the effect on plant growth. We observed that in the 12 mM Cu-amended treatment, all plants growth was stunted with leaves yellowing. In 16 mM Cu-amended treatment 5 out 8 plants were dead after 7 weeks of treatment, and the rest of the plants show stunted growth.

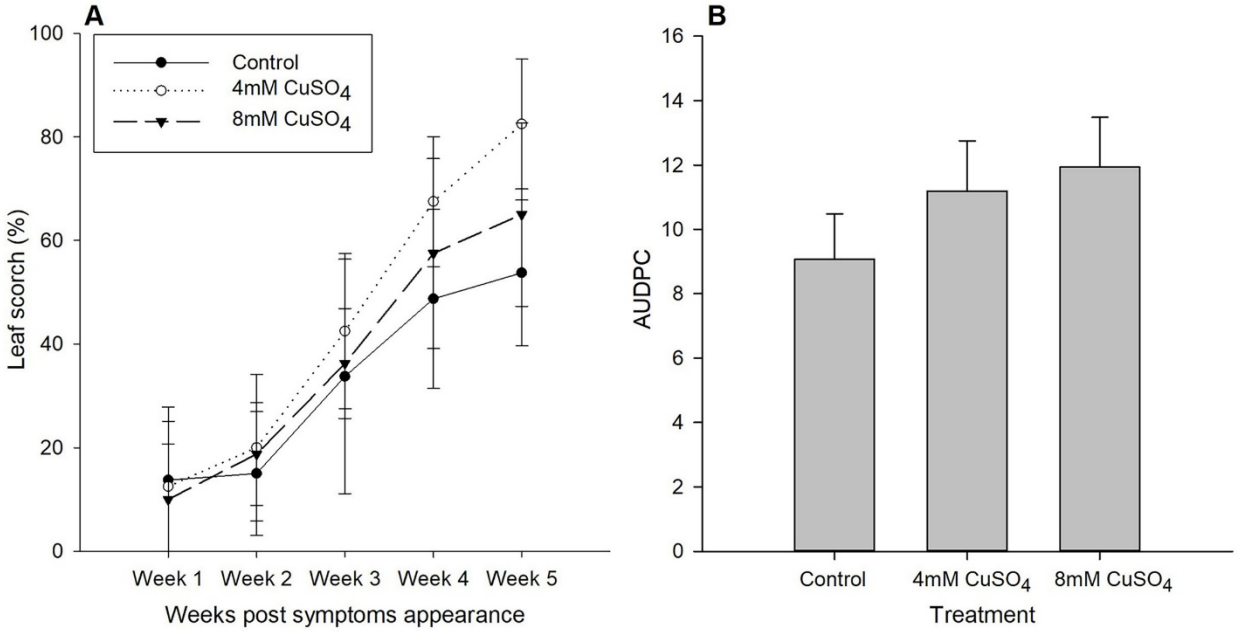

Fig. 4. Severity of *X. fastidiosa* caused symptoms with $CuSO_4$-amended treatments.

A, symptom development in tobacco plants. Disease severity is represented by the percentage of leaves showing scorch symptoms per plant. Eight plants were considered for each treatment. B, Area Under the Disease Progress Curve (AUDPC). $CuSO_4$-amended treatments showed a trend of increased disease symptoms compared to the control, but no significant differences were found between Cu-amended treatments and control, according to two-tailed Student's t-test (Microsoft Excel® 2016). Error bars in both A and B correspond to standard error of the means.

Data used for the graphs corresponds to one representative experiment. Three independent experiments performed under the same conditions showed similar tendencies.

X. fastidiosa populations in tobacco plants

qPCR and PMAxx-qPCR were used to quantify the total and viable populations of *X. fastidiosa,* respectively. There were no significant differences in bacterial total populations among plants of the control and Cu-amended treatments (Fig. 5). After 4 wps (Fig. 5A), the average total bacteria populations in plant leaf petioles of the control group was 5.75 ± 0.05 log CFU mg^{-1}. For $CuSO_4$-amended treatments the populations were 5.59 ± 0.03 log CFU mg^{-1} and 5.70 ± 0.03 log CFU mg^{-1} for 4 mM $CuSO_4$-amended treatment and 8 mM $CuSO_4$-amended treatment, respectively. At 8 wps (Fig. 5B) the average total bacterial populations were 5.22 ± 0.06 log CFU mg^{-1}, 5.30 ± 0.04 log CFU mg^{-1} and 5.41 ± 0.04 log CFU mg^{-1} for control group, 4 mM $CuSO_4$-amended treatment and 8 mM $CuSO_4$-amended treatment, respectively.

Viable bacterial populations were measured by PMAxx-qPCR. After 4 wps (Fig. 5A), the average viable bacteria populations in plant leaf petioles of control group was 3.08 ± 0.06 log CFU mg^{-1}, while for 4 mM $CuSO_4$-amended treatment was 3.25± 0.12 log CFU $mg^{-1}$and 8 mM $CuSO_4$-amended treatment was 3.22± 0.06 log CFU mg^{-1}. At 8 wps (Fig. 5B) the average viable bacterial populations were 3.64 ± 0.05 log CFU mg^{-1}, 4.02 ± 0.07 log CFU mg^{-1} and 4.05 ± 0.07 log CFU mg^{-1} for control group, 4 mM $CuSO_4$-amended treatment and 8 mM $CuSO_4$-amended treatment, respectively. At the later time point analyzed (8wps), viable populations increased significantly with amendments of $CuSO_4$ (Fig. 5B).

For assessment of the accuracy of the PMAxx treatment, a dead bacterial cell control was analyzed. After heat treatment of cells, PMAxx-qPCR still reported viable bacterial populations of 2.18 ± 0.09 log CFU mg^{-1}, while total population in the same sample assessed by qPCR was 4.46 ± 0.02 log CFU mg^{-1}.

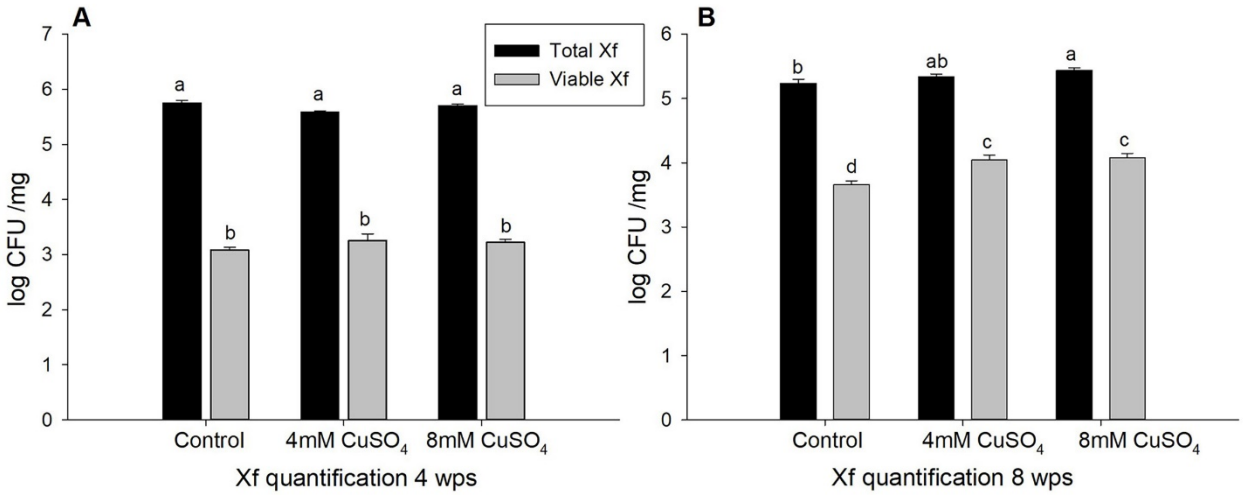

Fig. 5. Quantification *X. fastidiosa* in tobacco plant.

Bacterial populations were assessed at 4 wps (weeks post symptoms appearance) (A), and 8 wps (B). Total *X. fastidiosa* (Xf) population was measured by qPCR, and viable *X. fastidiosa* was measured by PMAxx-qPCR. Different letters indicates significant difference ($p < 0.05$) between treatments according to one-way ANOVA or Kruskal-Wallis. Error bars in both A and B correspond to standard error of the mean. Data used in the graphs corresponds to one representative experiment. Three independent experiments performed under the same conditions showed similar tendencies.

Discussion

In this study, we analyzed the effect of Cu accumulation in soil and subsequent uptake by roots of the plant host during *X. fastidiosa* infection. Our results show that Cu added as soil drench to tobacco plants caused an increment in Cu concentrations in sap and leaves, but this did not inhibit disease nor the pathogen growth in planta. Interestingly higher Cu concentration in sap seems to enhance virulence of *X. fastidiosa*, as noted by a non-significant trend of increase in the severity of disease symptoms when Cu was added to the watering solution, as well as an increase in bacterial populations.

Cu concentration of tobacco sap and leaves were both increased (40 to 100-fold changes in sap and 15 to 30-fold in leaves) when plants were drenched with $CuSO_4$ solution. This confirmed that Cu was successfully transported through the xylem system, and represents the situation of plants growing in soils with high Cu content. It has been shown previously that many plants, such as grapevine and citrus, are exposed to high Cu in their environment due to agricultural practices. For vineyards, Cu contained in soil ranges from 77 up to 3200 mg kg^{-1} (Mirlean et al. 2007; Ruyters et al. 2013). Grapevines that grow in a region where Cu content in surface soil (0-10 cm) is around 70 mg kg^{-1}, were shown to accumulate ~800 mg kg^{-1} Cu in leaves, and ~10 mg kg^{-1} in vascular tissue (Angelova et al. 1999). Similar Cu concentrations in grapevines were found in a study by Lai et al. (2010). Under Cu amendments, concentration in sap in our study was ~50 to 100 µM (3.0 to 6.6 mg kg^{-1} of sap fluid), and in leaves ~50 to 180 mg kg^{-1} of dried plant tissue. Therefore, our experimental setting represents conditions at the low end of the range of Cu found in grapevines around the world, particularly in sap where *X. fastidiosa* lives. In the studies mentioned above (Angelova et al. 1999; Lai et al. 2010) Cu was at much higher concentrations in leaves than in our experimental conditions, probably reflecting the fact that Cu was applied through leaf sprays in those studies; while in our research all Cu treatments were

applied as soil drench. Hippler et al. (2018) showed that in citrus trees, soil application and foliar application of Cu caused different Cu accumulation patterns. In soil applications, leaves Cu concentration ranged between 10-30 mg kg^{-1}, while this concentration went up to 600 mg kg^{-1} after foliar applications (Hippler et al. 2018). The majority of Cu applied in the field is through foliar sprays, since it is targeting foliar pathogens. While part of the Cu sprayed is taken by plants through leaves, most of the washed-off Cu stays on the soil surface, and will be accumulated year after year (Hippler et al. 2018; Mackie et al. 2012; Sun et al. 2018; Wightwick et al. 2008). Cu uptake and accumulation in plants could be crucial factors when considering plant disease management options related to Cu-containing antimicrobial agents. For *X. fastidiosa* management in the field, Cu-contaminated-soil needs to be taken into account due to the fact that vineyards, citrus orchards and other agriculture fields may have been through several decades of Cu applications (Wightwick et al. 2008). Cu concentration in those plants could benefit pathogen growth, especially after regulated uptake into the plant vascular system via plant homeostatic machinery.

When we measured disease development, we did not observe any reduction in disease symptoms in the Cu-amended treatments compared to controls, instead we noticed a tendency to show more symptoms at higher Cu treatments. Bacterial population were not reduced in Cu-amended treatments as compared to controls, instead they had significantly more viable cells at 8 wps. In this study, Cu concentration in tobacco sap was lower than 200 µM, which was the lowest concentration that significantly decreased *X. fastidiosa* biofilm formation and inhibited growth in our previous in vitro study (Cobine et al. 2013). In fact sap Cu concentration in Cu-amended treatments ranged from 10 to 100 µM, which is within the range that increased bacterial growth and biofilm formation in vitro (Cobine et al. 2013). A possible explanation for the tendency to

show more symptoms at higher Cu concentrations is increased biofilm formation in xylem, but this requires further investigation. Noteworthy is the observation that viable bacterial populations were higher in petioles of Cu-treated plants at later time points (8 wps), although it cannot be determined by our analysis if cells were forming biofilm. Also infected plants accumulated more Cu, which may have enhanced phytotoxicity. Our study treated plants with Cu only, as opposed to mixing Cu with Zn as in a recent field study concerned with suppression of olive quick decline syndrome (Scortichini et al. 2019). In that study, a Cu and Zn mixture was added to olive trees with a citric acid biocomplex (Dentamet®), and plants showed increase survival and decreased *X. fastidiosa* populations (Scortichini et al. 2019). Differences in the results may be due to different plant species tested in these studies, or bioavailability of Cu, however the concentration of leaf Cu was similar in both studies. We observed a much lower accumulation of Zn in our studies as compared to the study in olives (Scortichini et al. 2019), and the combination of Cu and Zn plus other components in the mixture may have contributed to the Dentamet® action.

Viable bacterial cells in plants were quantified by PMAxx-qPCR. To check the accuracy of the technique with plant samples, we used a control with heat-killed cells. In that control "live/viable" bacteria cells were still calculated at 2.18 log CFU mg^{-1} (4.46 log CFU mg^{-1} for total cells using regular qPCR), where 'dead cells' represented ~0.5% of total cells. Similar results described by others (Sicard et al. 2019) reported that for heat-killed *X. fastidiosa,* ~0.2-0.5% of cells had amplified DNA after PMAxx treatment. The authors suggested that some cells may be still alive after heat treatment, or the PMAxx treatment not reduced qPCR signal completely (Sicard et al. 2019). Other studies found that the efficiency of the dye depends on the plant species (Randazzo et al. 2016), and is influenced by other factors such as primers and polymerase used (Escudero-Abarca et al. 2014). Meanwhile, the incubation condition such as

buffer, light type and surfactants added could all influence the results (Fittipaldi et al. 2012; Moreno et al. 2015; Randazzo et al. 2016). In our study, we suggest that the viable cells measured by this method could be a reference to the number of viable cells in plant, but probably does not represent an absolute population value.

In this research, tobacco plants were grown in Cu-supplement soil, where plant Cu accumulation was significantly higher than controls watered with tap water, however, we did not notice symptoms related to Cu toxicity in plants (when drenched at 4 and 8 mM $CuSO_4$). Cu toxicity was observed when plants were watered with higher Cu concentrations (12 mM and 16 mM). Symptoms of Cu-induced phytotoxicity observed included chlorosis of the vegetative parts, reduced root growth, and photosynthesis alterations, as previously described (Culotta and Scott 2016). Plants Cu homeostasis help avoid excess of Cu and therefore prevents toxicity. Cu transporters play an important role in regulating Cu content in the cytosol. ATP-independent Cu transporters COPTs (copper transporters), YSL (yellow stripe-like) and ZIP (ZRT-IRT-like transporter) are involved with Cu import into cytosol, while P_{1B}-type ATPases are related to Cu export outside of the cytosol (Kampfenkel 1995; Pilon 2011; Puig et al. 2007). Once in the cytosol metallothioneins and phytochelatins can bind Cu to decrease free Cu ion in plant cells (Burkhead et al. 2009). Recent research showed that sap leakage from the trunk and twigs was a strategy of plants to reduce accumulation of Cu after high dosages of this element (Hippler et al. 2018). We hypothesize that a functional plant Cu homeostasis restricts the uptake of Cu to a tight range, and *X. fastidiosa* takes advantage of this set point to gain sufficient Cu from sap for its own growth, which leads to more bacterial growth, biofilm formation and more severe disease.

Cu treatments to control plant diseases have been applied in the field for more than a century, in particular the Bordeaux mixture containing Cu-sulfate combined with Ca-hydroxide. The long-

term usage of these management strategies have led to Cu accumulation in field soils and now we must consider the possible consequences it might cause. *X. fastidiosa* appears to be able to grow under Cu treatment here by soil drench, and we have shown extensively that Ca enhances the virulence traits of *X. fastidiosa* strains (Cruz et al. 2012; Parker et al. 2016). Cu from polluted soils could serve as booster for endophytic pathogen growth, as we shown in this study. This is a reminder that plant disease management in the field should not just focus on the efficiency of controlling one single pathogen, but also the influence to non-target microflora.

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Chapter 3: Copper homeostasis genes *copA* and *copB* influence virulence and biofilm formation by the plant pathogenic bacterium *Xylella fastidiosa*

Abstract

Xylella fastidiosa is a xylem-limited plant pathogenic bacterium that causes diseases worldwide in crops such as grape, citrus and olives. Although copper (Cu)-containing compounds are not used for management of *X. fastidiosa*-caused diseases, these are widely used in *X. fastidiosa* hosts in vineyards and orchards. The accumulation of Cu in soils, and therefore plant vascular systems, could be a challenge for *X. fastidiosa* survival. Here, the molecular basis of Cu homeostasis was studied in relation to virulence. Although homologous Cu-related genes *copA* (*X. fastidiosa* loci PD0100) and *copB* (PD0101) have been characterized in other bacteria, their function differs among bacterial species. Both *copA* and *copB* mutants of *X. fastidiosa* were more sensitive to Cu than the wild-type (WT) strain. Interestingly, the *copA* mutant was more sensitive to Cu shock, while *copB* mutant was more sensitive to chronic Cu treatments. Greenhouse experiments showed that under normal watering regime, both *copA* and *copB* mutants caused reduced virulence compared to WT. But when Cu was added as a drench treatment, both mutants increased disease severity ~20% and ~50%, respectively; significantly higher increments than the ~5% observed for WT under the same conditions. These results indicate that the pathogen's Cu homeostasis effect on virulence is influenced by the Cu concentration in the environment. Understanding Cu homeostasis in *X. fastidiosa* will help discern the outcome of possible Cu treatments and the adaptation of this pathogen to the xylem environment of plants that have been exposed to high Cu concentrations due to agricultural practices.

Introduction

Copper (Cu) is widely distributed in the environment and it is an essential micronutrient that is required by all living organisms (Zhao et al., 2014). The main functions of Cu is to serve as co-factor in metalloproteins that maintain normal metabolism (Brown et al., 1992). However, due to redox cycling between Cu(I) and Cu(II) and inappropriate binding to proteins, Cu is toxic to organisms at high concentrations. Cu redox cycling causes protein oxidization, as well as lipid, nucleic acid, and polysaccharide structure alterations (Brown et al., 1992, Rensing & Grass, 2003). For this reason, Cu has been used as a powerful chemical control in agriculture for many years (Brun et al., 1998). Fungal diseases such as downy mildew, and bacterial diseases such as bacterial spot of tomatoes, can be controlled by Cu-containing chemical treatments in the field (Brun et al., 1998, Rusjan, 2012, Itako et al., 2015). These spray treatments cause both a sudden influx of Cu (Cu shock) to the pathogen and also cause increased residual Cu to accumulate on the leaves and in the soil (chronic Cu-exposure).

The Gram-negative bacterial pathogen *Xylella fastidiosa* causes diseases such as Pierce's disease (PD) of grapevine and bacterial leaf scorch (BLS) of blueberry (Purcell & Hopkins, 1996, Hopkins & Purcell, 2002, Oliver et al., 2015). The vineyard and orchards where it is found have widespread use of antimicrobial Cu-compounds (Komárek et al., 2010, Brunetto et al., 2016, Ballabio et al., 2018). Soil from many vineyards and orchards often contain Cu that exceeds the allowed maximum residue level (200 mg/kg of Cu in soil) (Komárek et al., 2010, Brunetto et al., 2016). Our previous study showed that when plants were exposed to increased soil Cu concentration this leads to accumulation of Cu in plants, but this was unsuccessful at controlling diseases progression caused by *X. fastidiosa*. Instead, high Cu concentration in plants

tends to increase disease symptoms (Ge et al., 2020). Molecular mechanisms of how *X. fastidiosa* manages the increased Cu in the plant while it maintains Cu homeostasis are unknown.

Bacteria can have multiple Cu-responsive systems, including chromosomal and plasmid Cu-resistance systems, to help them survive under high Cu environments and maintain Cu homeostasis (Brown et al., 1992). The plasmid-borne Cu resistance operon *pco* system, which contains seven genes *pcoABCDRSE*, was first described in *E. coli* conjugative plasmid pRJ10004 (Rouch & Brown, 1997, Huffman et al., 2002, Lee et al., 2002). Homologous genes are found in plasmid and chromosome of *Pseudomonas* spp., *Xanthomonas* spp. and other bacteria (Cha & Cooksey, 1991, Cooksey, 1993, Teixeira et al., 2008, Behlau et al., 2011). Homologs of *pco* genes (*cop* genes) are present in the chromosome of *X. fastidiosa* and their function was studied here. The mechanisms of Cu resistance and homeostasis vary even in bacteria where the genes are homologous especially in plant pathogens (Bondarczuk & Piotrowska-Seget, 2013). Here we used molecular methods and greenhouse experiments to understand Cu homeostasis in *X. fastidiosa*. We found that both *copA* and *copB* play important roles in biofilm formation and virulence in planta, particularly under high xylem Cu concentrations.

Materials and Methods

Bacterial strains and culture conditions

Xylella fastidiosa subsp. *fastidiosa* type strain TemeculaL was used as the wild-type (WT) strain in this study. WT and mutants were grown at 28°C using Periwinkle Wilt (PW) agar plates

(Davis et al., 1981), Pierce's Disease 2 (PD2) broth (Davis et al., 1980) or PD3 medium (Davis et al., 1981) with or without kanamycin (Km) (50 µg/ml). Bacterial suspensions of *X. fastidiosa* for plant inoculation experiments were prepared as follows. *X. fastidiosa* was streaked from -80 ℃ frozen glycerol (20%) stocks on PW agar plates (Km was added for mutants) for 5-7 days, and then re-streaked onto new plates for additional 5-7 days. Bacterial cultures were suspended in PBS (phosphate-buffered saline) for plant inoculations. Bacterial suspensions of *X. fastidiosa* for in vitro experiments were prepared similarly as mentioned above, but the suspensions were made using PD2 broth instead of PBS.

Construction of mutant strains

Natural competence and homologous recombination were used to generate *X. fastidiosa copA* (PD0100) and *copB* (PD0101) null mutants, following a previously-described protocol (Kandel et al., 2018). Briefly, *X. fastidiosa* WT genomic DNA was extracted by using a modified CTAB protocol (Doyle and Doyle 1987). Then, ~1,000bp upstream and downstream of each *copA* and *copB* open reading frames (ORF) were amplified by PCR. Primers used for all amplifications are listed in supplemental Table 1. A Km cassette was amplified from pUC4K with primers that have ~21 bp overlapping sequences with target gene upstream and downstream sequences. PCR products were purified with Gel purification kit (Zymo Research, Irvine, CA) for overlap extension PCR performed by using three PCR fragments: gene upstream, downstream and Km cassette as templates to obtain one fusion PCR fragment. The fusion PCR fragments were purified and mixed with 10-µl cell suspension [optical density $(OD)_{600nm}$=0.25] of WT strain on PD3 agar plates. After 3 days of co-culture, bacteria were suspended in 200 µl of PD3. 100 µl

and 20 µl of bacterial suspensions were plated onto PW+Km plates for selection. After ~2 weeks, single colonies were pick and re-streaked to new PW+Km plates. PCR and sequencing were carried to confirm the mutants (data not shown). PCR was performed with a standard protocol, using an iProof High-Fidelity PCR kit (Bio-Rad, Hercules, CA) in an S1000 thermal cycler (Bio-Rad, Hercules, CA).

Planktonic growth and biofilm formation in media with different Cu concentrations

Biofilm formation and planktonic growth of *X. fastidiosa* WT and mutants were quantified in 96-well plates as previously described (Cruz et al., 2012, Chen et al., 2017), with some modifications. Briefly, bacteria (initial OD_{600nm}= 0.01) were grown in 96-wells plates with 200µl of PD2 broth alone or amended with 4 different concentrations of $CuSO_4$ (50 µM, 100 µM, 150 µM and 200 µM) on each well. Plates were shaken at 140 rpm for 5 days. Then, 150 µl of supernatant of bacteria culture were removed to new 96-wells plates, and OD_{600nm} measurements were used to calculate planktonic growth. The original culture plates were rinsed two times with Milli-Q water (Millipore, Burlington, MA), and dyed with 230 µl of 0.1% crystal violet for 20 min. Then, crystal violet was removed, and plates were rinsed 3 times with Milli-Q water. Finally, 230 µl of 95% ethanol were added and incubated for 5 to 10 min. OD_{600nm} values were used to calculate biofilm formation. Twenty-four repetitions per treatment were used for each independent experiment, and 3 independent experiment were carried in this study.

Gene expression of *copA* and *copB* genes

Gene expression of *copA* and *copB* was analyzed under Cu-replete (100 µM, 200 µM, 250 µM and 350 µM $CuSO_4$) and Cu-depleted conditions (150 µM BCS: bathocuproine sulfate). *X. fastidiosa* WT cells were grown in 5ml PD2 media with initial OD_{600nm}=0.01. After 7 days, bacteria were collected by centrifugation at 13,000x g for 3 min. Total RNA from each treatment was extracted by using the Quick-RNA MiniPrep kit (Zymo Research, Irvine, CA). Contaminant DNA in samples was removed by treatment with DNase I (Thermo Scientific, Pittsburgh, PA). One-µg RNA of each sample was used to synthesize single-strand cDNA by reverse transcription using the Maxima First Strand cDNA synthesis kit for reverse transcription-quantitative PCR (RT-qPCR) (Thermo Scientific, Pittsburgh, PA). cDNA samples were diluted 5 times with RNA/DNA free water. Primers used for *copA* and *copB* were designed using PrimerQuest software (Integrated DNA Technologies). *gyrB* was used as internal control, as previously described (Cruz et al., 2014). The qPCR was carried with SYBR green (Bio-Rad, Hercules, CA) by ABI 7500 real-time PCR system (Applied Biosystems, Foster City, CA). The fold change in gene expression compared with WT was calculated according to the $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen, 2001).

Mutant population quantification under Cu shock

X. fastidiosa WT and mutants were grown in 5ml PD2 media with initial OD_{600nm}= 0.01. After 3 days of culturing, $CuSO_4$ was added to a final concentration of 500 µM as a Cu shock, while same volume of PD2 media were added to control. After 12h of Cu shock, bacteria were collected by centrifugation at 4,000x g for 10 min at 4°C, then re-suspend onto 1 ml PD2 media. From the original bacterial suspension, 10^{-1} to 10^{-6} dilutions were made for each strain and plated

onto PW (n=8) for 10 to 14 days culturing. Bacterial population of each strains with and without Cu treatment was calculated by CFU counting. Three independent experiments were carried out in this study.

Bacteria growth under Cu-amended conditions in microfluidic chambers

Microfluidic chambers (MC) are a system that allows microscopic imaging of bacteria cells under continuous flow culture conditions, and mimic microscale vascular tissue of plants (De La Fuente et al., 2007). MC were fabricated as previously described (De La Fuente et al. 2007) Mutants' growth under Cu-amended condition was assessed in MCs. Briefly, *X. fastidiosa* WT and *copA*/*copB* mutants were suspended in PD2 broth (OD_{600nm}=0.8) and placed in 1-ml plastic syringes used for bacteria inlets. Ten-ml plastic syringes were filled with PD2 broth or PD2 with 100μM $CuSO_4$ used as media inlets for bacterial culture. Both media and bacterial suspensions were pumped into the microchannels by an automatic syringe pump (PicoPlus, Harvard Apparatus, Holliston, MA), with controlled flow rate. Nikon Eclipse Ti inverted microscope (Nikon, Melville, NY) (40x objective) were used to observe and record MCs. To start MC experiments, bacterial suspension was pumped into MCs until bacteria cells were observed in chambers and filled ~ ¾ of chamber surface. Media flow speed was set at 0.25 μl/min for continuously bacteria culture. Nikon DS-Q1 digital camera (Nikon, Melville, NY) was used to capture time-lapse microscopy images every 2 min, controlled by NIS-Elements Advanced Research 3.01 (Nikon, Melville, NY). The growth of mutants was compared with WT under MC conditions for 7-9 days. After that, bacteria were stained with LIVE/DEAD® BactLight Bacterial Viability Kit following a previously developed protocol (Naranjo et al., 2020). Dye mixture was

diluted with PD2 to a final concentration of 100 nM. The media inputs of chambers were gently replaced (avoiding creating bubbles) by the dye-PD2 mixture. After 12 h pumping media with 0.25 µl/min flow speed, fluorescence pictures of the chamber were recorded by Nikon DS-Q1 digital camera (Nikon, Melville, NY).

Greenhouse experiments

X. fastidiosa mutants virulence was studied using the model plant tobacco (*Nicotiana tabacum* 'Petite Havana SR1', plant introduction number 552516), which was grown and prepared for inoculation as previously described (Francis et al., 2008, De La Fuente et al., 2013, Ge et al., 2020). Briefly, upper leaves of 6-weeks-old tobacco plants were trimmed and only three healthy lower leaves were kept. Bacterial inoculum was prepared as described above (OD_{600nm}=0.8 suspended in PBS). Small wounds were punctured at the base of the leaf petioles using a 23-gauge needle. Consequently, 20 µl of bacteria inoculum were added on top of the small wounds for plants to take up. Eighteen plants were treated for each bacterial strain. Plants injected with PBS buffer only were consider as control. Second inoculation carried after one week of first inoculation with same method. One new shoot per plant was kept for later growth. For each bacterial strain treatment, 9 plants were treated with 200 mL 8 mM $CuSO_4$, and the other 9 plants with tap water (Ge et al. 2020). Treatments with additional Cu started one day after the first inoculation and repeated once a week. Eighty-90 days after the first inoculation, symptoms (leaf scorch) started to show. The number of leaves showing scorch symptoms per plant was recorded as scorch leaves/total leaves and expressed as percentage of leaf scorch and used as measurement of disease severity (Lindow et al., 2014). The area under the disease progress curve (AUDPC)

was calculated based on the midpoint rule method (Campbell & Madden, 1990) as follows: AUDPC= Σ [(yi + yi+1)/2](ti+1 – ti), where i = the number of assessment times, y = disease severity score for each plant at each assessment, and t = time at each assessment. A disease severity score scale was used for AUDPC calculation: leaf scorch symptoms on each leaf were given scores (0-5) according to the following scale: 100%, 75%, 50% and 25% of leaf surface area with scorch symptoms were considered as 5, 4, 3 and 2 score, respectively. Only the tip part of leaves with scorch symptoms were considered as 1 score, and no symptoms on leaves was 0 score.

Ionome characterization

Mineral elements concentration in bacterial cells was measured by ICP-OES (Inductively Coupled Plasma with Optical Emission Spectroscopy) (Perkin Elmer 7100 DV, Waltham, MA). Bacteria were grown in PD2 with either 0 µM, 20 µM or 50 µM $CuSO_4$ (initial OD_{600nm}=0.01). After 7 days of incubation with shaking at 140 rpm, bacterial cells were collected by centrifugation at 10,000x g for 3 min. The pellet was washed twice by resuspension in Milli-Q water followed by centrifugation. For each sample, 100 µl of mineral-free concentrated nitric acid (OPTIMA, Fisher Scientific) were added for digestion and treated for 1 hour at 100 °C. Later, 200 µl Milli-Q water were added to each sample and analyzed by ICP-OES with simultaneous measurement of Ca, Fe, Cu, Zn, Mn, S, Mg, K, Na, and P. Mineral elements concentration were determined by comparing emission intensities to standard curves created from certified standards (SPEXCertiprep, Metuchen, NJ). Standard curves were confirmed by reanalysis of standard solutions diluted in a matrix equivalent to the sample. Individual readings

(average of two intensity measurements) and repeated analysis of individual samples, showed less than 5% variation (data not shown).

For leaf ionome characterization, 5 plants were selected from each treatment and 5 leaves above the inoculation point of each selected plant were collected. Ionome characterization was carried by ICP-OES as previously-described (De La Fuente et al., 2013, Ge et al., 2020). Briefly, leaves were collected and dried at 80 ℃ for 1 hour, and then ground to fine powder. Ten-mg of leaves powder were digested with mineral-free concentrated nitric acid and diluted with Milli-Q water. Remaining particulates were removed by centrifugation before running the samples in ICP-OES.

Sap from 5 plants that were selected in each treatment were collected for ionome analysis. The sap collection was performed using Scholander pump model 600 pressure chamber (PMS Instrument Company, Corvallis, OR) as described previously (Oliver et al., 2014). Sap samples could be used directly for ICP-OES analysis.

Total and viable bacterial population quantification

To quantify the total and viable population of *X. fastidiosa* mutants in plant, quantitative polymerase chain reaction (qPCR) and PMAxx (Biotium, Hayward, CA, USA) were used, respectively, as previously described (De La Fuente et al., 2013, Ge et al., 2020). Briefly, leaf petioles were collected at the end of symptoms evaluation. Around 100 mg-fresh petioles were cut into small pieces and ground by using a mini Beadbeater 96 (Biospec Products, Bartlesville, OK) with 2.0 mm beads (ZIRCONIA, Biospec Products, Bartlesville, OK) for 2-3 min at the highest speed. Then, 1.5 ml of PD2 broth were added to samples and mixed thoroughly. From

there, 500 µl-sample was used for total bacteria population quantification. A second 500-µl sample was treated with PMAxx (final concentration of 50 µM) for viable bacteria population quantification. The samples were incubated in the dark for 5 min, and then exposed to a strong white light (700W halogen portable work light, Husky Co., Atlanta, GA) for 10 min with ice placed below to prevent overheat and occasionally mix. Modified CTAB protocol (Doyle & Doyle, 1987) was used to extract DNA from total and viable bacteria samples. Quantification of *X. fastidiosa* populations were carried by qPCR following a previously described protocol (De La Fuente et al., 2013).

Statistical analyses

For gene expression, biofilm formation, culturable bacteria population, disease symptoms and AUDPC, statistical analyses were determined by the two-tailed Student's *t-test* (p <0.050) (Excel 2016 for Windows). For the analysis of Cu concentrations in mutants, sap, leaf ionomes and bacterial population quantification, pairwise multiple comparison procedures using Tukey's test (Kruskal Wallis for non-normal data) using SigmaPlot 14.0 (Systat Software, Inc. SigmaPlot for Windows) (p <0.050). Principal Component Analysis (PCA) and heat map were prepared using ClustVis (Metsalu & Vilo, 2015).

Results

Gene expression of *copA* and *copB* genes

To confirm a role in Cu-response, gene expression of *copA* and *copB* in *X. fastidiosa* TemeculaL (WT) was assesed under different Cu concentrations. Expression of both genes was increased when Cu concentration was increased from 0 to 250 µM, while it decreased at 350 µM Cu. When *X. fastidiosa* cells were grown under Cu deficiency condition (150 µM BCS: bathocuproine sulfate), expression of both *copA* and *copB* genes was decreased (Fig. 1).

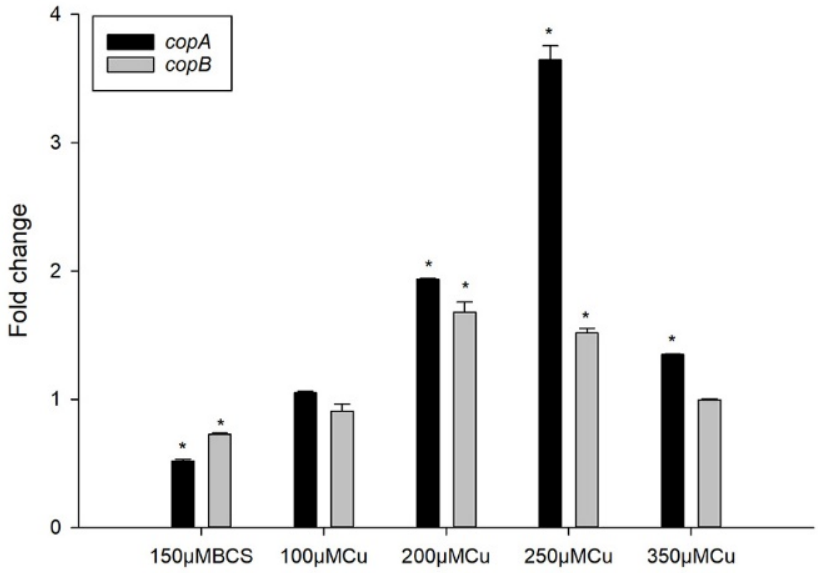

Fig. 1. Gene expression of *copA* and *copB* genes

X. fastidiosa WT cells were grown in test tubes for 7 days in PD2 media amended with different concentrations of $CuSO_4$ or the Cu chelator BCS. Gene expression was measured by RT-qPCR, using *gyrB* as internal control. Fold change of gene expression were compared with that of unamended PD2 media. Mean values of 3 independent repetitions (n= 9) are shown in graph, and error bars represent standard error of the mean. * indicate significant differences from the unamended PD2 treatment ($p < 0.05$) in gene expression as determined by the two-tailed Student's *t-test*.

Effect of *copA* and *copB* mutations on planktonic growth and biofilm formation by *X. fastidiosa* under Cu amendments.

To determine the growth characteristics of *copA* and *copB* mutants, planktonic growth and biofilm formation of both mutants were analyzed under Cu-amended conditions. Four concentrations of $CuSO_4$ (50 µM, 100 µM, 150 µM and 200 µM) were tested and compared against growth in non-amended PD2 (Pierce's Disease 2) media. Mutants showed similar trends as WT in Cu-amended treatment with increasing biofilm formation at lower Cu concentration before eventually decreasing at higher Cu concentration (Fig. 2). However, the threshold Cu concentration was markedly different between WT and the mutants. WT had the highest biofilm formation between 100-200 µM Cu, which increased around 230% as compared to non-amended media (Fig. 2). On the other hand, *copA* and *copB* mutants had the highest biofilm formation at 50 µM Cu, which increased ~ 200% for *copA* mutant and 180% for *copB* mutant (Fig. 2). Biofilm formation of mutants decreased at much lower Cu concentration than that of WT. Interestingly, Cu-amended treatments had little influence on planktonic growth of WT and mutants (Fig. 2). In summary, biofilm formation by *copA* and *copB* mutants was more sensitive to Cu than WT.

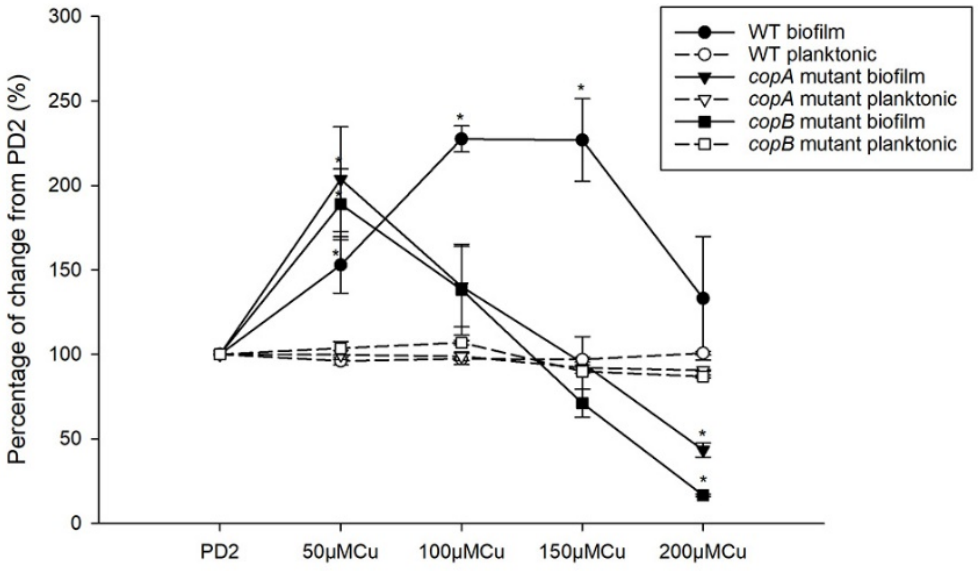

Fig. 2. Biofilm formation of *copA* and *copB* mutants

Mutants and WT were grown in 96-wells plates with PD2 media amended with different concentrations of $CuSO_4$. Biofilm was assessed by OD_{600} of crystal violet staining. The value of 100% represents the growth of bacteria in PD2 media without any amendments, and was used as control. Numbers above 100% represent growth increase, and below 100% represent growth inhibition. * indicate significant differences ($p < 0.05$) in OD_{600nm} values between $CuSO_4$-amended treatments and PD2 as determined by the two-tailed Student's *t-test*.

Cu accumulation in mutants under Cu treatment

To characterize potential function of *copA* and *copB* Cu accumulation in mutant cells was measured by ICP-OES (Inductively Coupled Plasma with Optical Emission Spectroscopy) and compared with WT. Significantly higher Cu accumulation was found in *copA* and *copB* mutants compared to WT ($p < 0.05$) at both 20 μM and 50 μM Cu conditions. When cultured in PD2 media, mutants did not show significant differences with WT ($p = 0.413$) (Fig. 3).

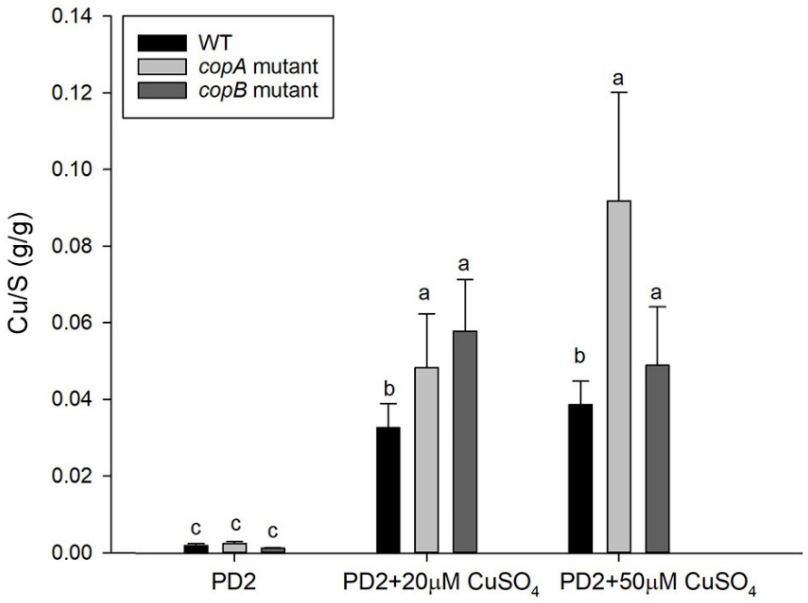

Fig. 3. Cu accumulation in *copA* and *copB* mutants.

Mutants and WT were grown for 7 days in test tubes with PD2 media amended with 20μM or 50μM $CuSO_4$. Cu content in cells was measured by ICP-OES. Mean values are shown in graph, and error bars represent standard error of the mean. Data used in the graph corresponds to one representative experiment, and three independent experiments performed under the same conditions showed similar tendencies. Different letters above bars indicate significant differences ($p < 0.05$) among all treatments according to Tukey's test (all pairwise multiple comparison procedures).

Resistance of *copA* and *copB* mutant to Cu shock

Viable populations of the WT and mutants were assessed after exposure to Cu shock of 500 μM Cu for 12 h after OD (optical density) reached ~0.1. WT culturable population (measured as CFU on agar plates) decreased 7-fold after Cu shock (Table 1) while the culturable population of *copA* mutant under the same Cu treatment was decreased by ~100 fold, showing a significant

difference (p =0.010) in resistance to Cu. For *copB* mutant, the population decreased by 15 times compared to its control, which was also significantly different ($p < 0.001$) to WT but the magnitude of change was smaller than *copA*. *copA* seems to be more important than *copB* for *X. fastidiosa* to withstand Cu shock. Cu accumulation in mutants under Cu shock was measured by ICP-OES (Fig. 4). Cu accumulation significantly increased in WT and mutants. Significant difference ($p = 0.03$) were found between *copB* and *copA* mutants in Cu accumulation after acute Cu treatment. Other than the changes in Cu no significant ($p > 0.05$) differences in other elements tested between WT and *copB* or *copA* mutants were detected.

CFU/ml	Control	500µM Cu	Times decreased
WT	$2.4x10^8$	$3.5\ x10^7$	7.0X
***copA* mutant**	$3.1x10^8$	$2.9\ x10^6$	109.7X*
***copB* mutant**	$3.3x10^8$	$2.2\ x10^7$	15.1X*

Table 1. Culturable bacterial population (CFU) changes after Cu shock

Bacterial cells were grown in PD2 liquid media prior to an acute Cu treatment (500µM for 12h) was added. Culturable bacteria was quantified as CFU by plating in PW agar plates after Cu treatment and mock treatment (control) * indicate significant differences ($p < 0.05$) in CFU reduction between mutants and WT as determined by two-tailed Student's *t-test*.

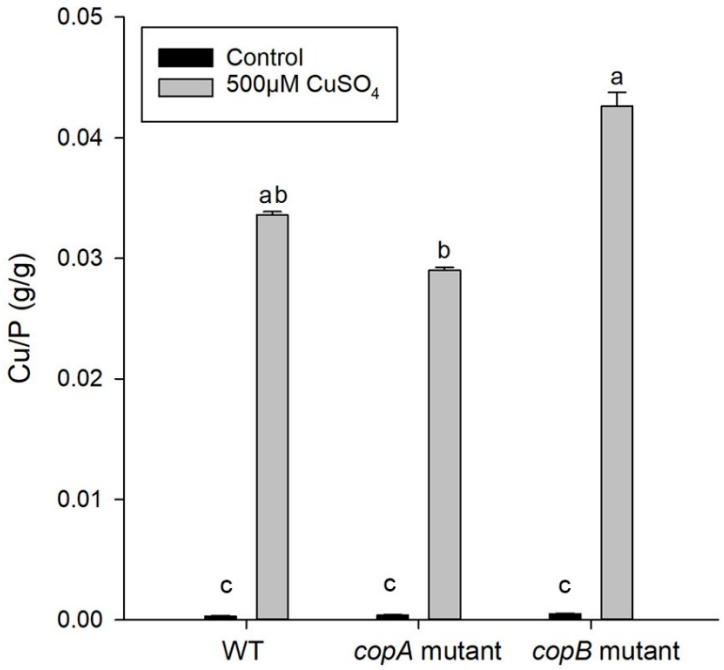

Fig. 4. Cu content in *copA* and *copB* mutants after Cu shock

Mutants and WT cells were collected after Cu shock. Cu content in cells was measured by ICP-OES. Bars in graph represent mean values, and error bars represent standard error of the mean. Different letters above bars indicate significant differences ($p < 0.05$) among all treatments according to Kruskal-Wallis one way analysis of variance on ranks.

Growth of *copA* and *copB* mutants in microfluidic chambers

Mutants were grown under flow conditions that more closely resemble xylem vessels in microfluidic chambers (MC) using PD2 with and without addition of 100 µM Cu. Mutants and WT were cultured 7 days with a media flow speed of 0.25 µl/min. The growth of both *copA* and *copB* mutants was decreased after 7-day culturing as compared to WT (Fig. 5). Observable cell movement stopped around 5 to 6 days for *copA* mutants and around 4 days for *copB* mutants.

Both cell-to-cell aggregation and biofilm formation of mutants were inhibited under 100µM $CuSO_4$. After 7 days of growth under Cu-amended media, bacteria were stained with LIVE/DEAD® BactLight Bacterial Viability Kit, which stains live cells green and dead cells red. WT had both live and dead cells with profuse biofilm formation (Fig. 5). *copA* mutant had both live and dead cells, however, unlike WT, cells did not form biofilm but were observed as single cells (Fig. 5). For *copB* mutant, most of the cells were dead, with no significant presence of live cells in the whole MC (Fig. 5). No biofilm formation was observed with the *copB* mutant.

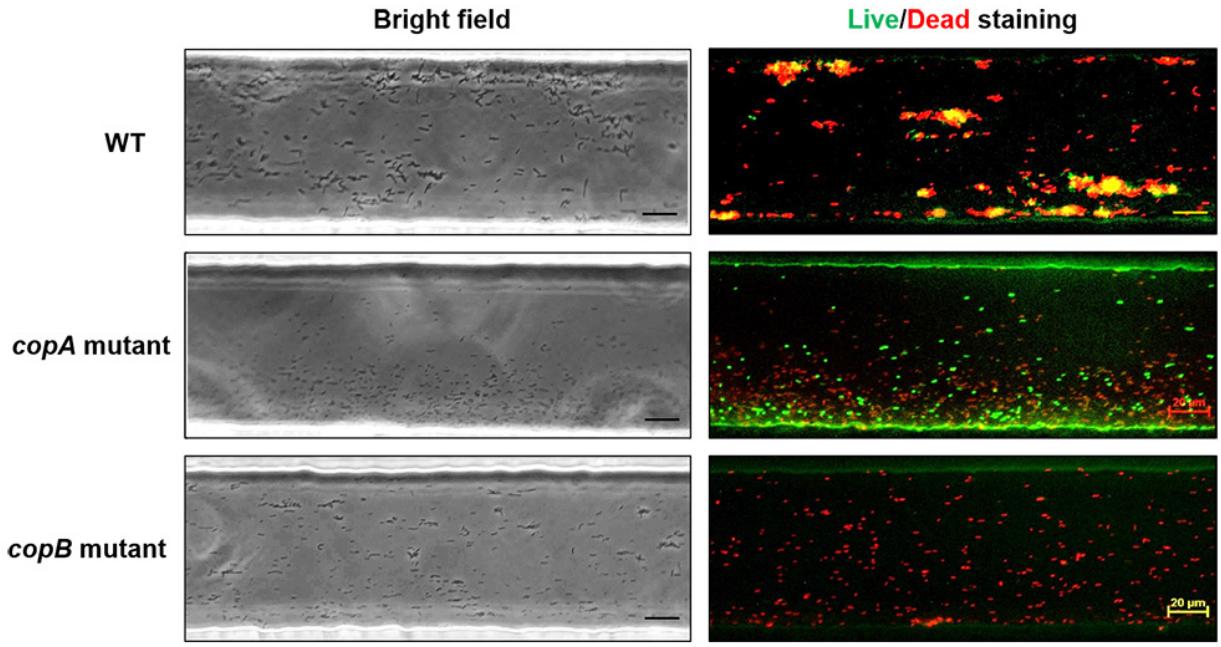

Fig. 5. Growth of WT and mutants in 100µM $CuSO_4$ in microfluidic chambers

Mutants and WT were grown in microfluidic chambers for with PD2+100µM $CuSO_4$ media. Bright field pictures were taken at the end of 7th day. Bacteria were stained at 8th day with LIVE/DEAD® BactLight Bacterial Viability Kit. Live cells stained green while dead cells stained red. Scale bar: 20µm.

copA and _copB_ mutants cause more severe disease symptoms with Cu-amended treatments

Disease symptoms of leaf scorch in tobacco plants started to show after 75-80 days post-inoculation with WT and mutant *X. fastidiosa*. Five weeks after initial symptoms appeared WT showed approximately 63% of leaves with scorch symptoms with water treatment (control), and 69% with 8 mM $CuSO_4$-amended treatment (Fig. 6a). For *copA* mutant-inoculated plants, around 40% leaf scorch was shown under water treatment; while more than 60% of leaves showed scorch symptoms with 8 mM $CuSO_4$-amended treatment (Fig. 6a). For *copB* mutant-inoculated plants, the gap between water treatment and 8 mM $CuSO_4$-amended treatment was higher than that of *copA* mutant-inoculated plants, around 40% for water treatment and around 90% for 8 mM $CuSO_4$-amended treatment (Fig. 6a). Disease symptoms of mutants-inoculated plants dramatically increased with 8 mM $CuSO_4$-amended treatment. Increment in severity for *copA* mutant was ~20% compared to unamended watering control treatment, for *copB* mutant was ~50%, and both were more pronounced than WT, which had an increment in severity of ~5% (Fig. 6a). The area under the disease progress curve (AUDPC) was significantly ($p < 0.01$) increased in *copB* mutant with 8 mM $CuSO_4$-amended treatment compared with water treatment (Fig. 6b). For *copA* mutant a clear tendency of increment of AUDPC, although not statistically significant ($p = 0.12$), was noticed in 8 mM $CuSO_4$-amended treatment compared with water treatment (Fig. 6b). Total and viable population of WT and mutants were quantified by qPCR and PMAxx-qPCR, respectively. No significant difference in bacteria populations were observed for both mutants with and without $CuSO_4$-amended treatment compared to WT (Supplemental Fig. 1). Total bacteria population of mutants and WT were all around 5.5 log CFU/mg. The viable bacteria population was around 4 log CFU/mg.

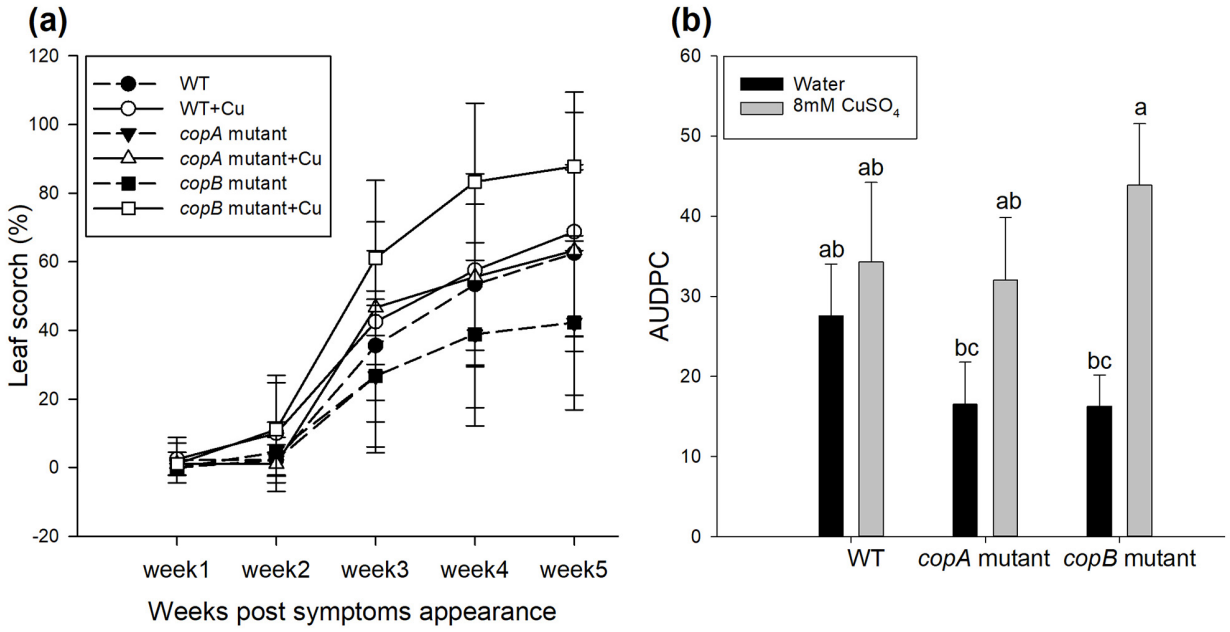

Fig. 6. Severity of mutants caused symptoms with CuSO4-amended treatments

Symptom development in tobacco plants. (a): Disease severity is represented by the percentage of leaves showing scorch symptoms per plant (leaf scorch % = (#scorched leaves*100)/#total leaves). (b) AUDPC= Σ [(yi + yi+1)/2](ti+1 – ti), where i = the number of assessment times, y = total score of leaf scorch in each plant at each assessment, and t = time at each assessment. Eight plants were considered for each treatment. Error bars correspond to standard error of the means. Data used for graph corresponds to one representative experiment. Three independent experiments performed under the same conditions showed similar tendencies. Different letters above bars in B indicate significant differences ($p < 0.05$) among all treatments according to two-tailed Student's *t-test*.

Mutants increased Cu content in plants sap but not in plant leaves

The sap and leaf ionome of plants infected with *copA*, *copB* mutants or WT under different Cu concentrations was measured using ICP-OES. $CuSO_4$-amended treatments in plants increased Cu

content as previously reported (Ge et al., 2020). Cu content in sap from plants under unamended water treatment (black bars) was higher in both mutants compared to the uninoculated control and WT-inoculated plants (Fig. 7a).

When 8 mM $CuSO_4$ was used as watering solution, Cu content in sap of plants inoculated with *copB* mutant was higher than uninoculated plants and WT or *copA* mutant-inoculated treatments, although non-significant differences were found with the last two treatments. Plants inoculated with *copA* mutant were not statistically significantly different from either uninoculated or WT control (Figure 7a). When control plants were treated with water, there was no difference between mutants and WT in Cu content in leaves (Figure 7b). When leaf Cu content was quantified for 8 mM $CuSO_4$-amended treatments, *copA* and *copB* mutants showed similar trends to what was found for sap, however no statistical significance was found among treatments (Figure 7b).

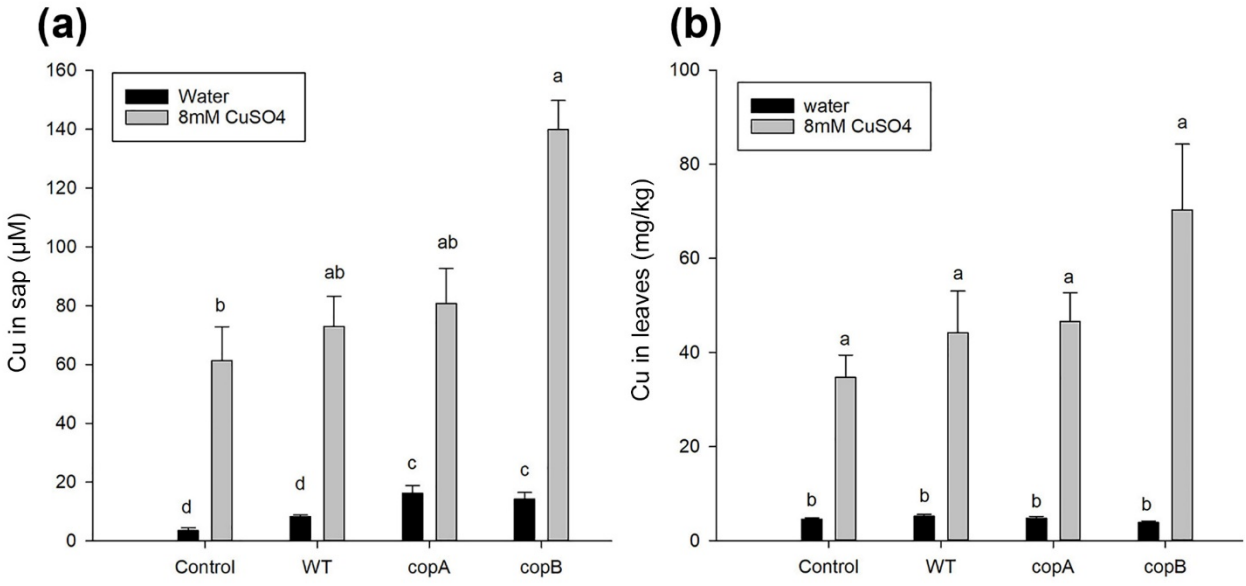

Fig. 7. Cu concentration in sap (a) and leaves (b) of tobacco plants

Sap and leaves samples were collected from tobacco plants grown in the greenhouse (n = 5/treatment). Plants were inoculated with PBS ('Control'), *X. fastidiosa* WT ('WT'), *copA* mutant ('copA') and *copB* mutant ('copB'). Plants were watered either with 8mM $CuSO_4$ or tap water (see methods). Mean values are shown in graph, and error bars represent standard error of the mean. Data used in the graph corresponds to one representative experiment, and three independent experiments performed under the same conditions showed similar tendencies. Different letters above bars indicate significant differences ($p < 0.05$) among all treatments according to Tukey's test (all pairwise multiple comparison procedures).

***copA* and *copB* mutants affect ionome in tobacco leaves differently than WT**

Leaf ionome (Ca, Cu, Fe, K, Mg, Mn, Na, P and Zn) was measured by ICP-OES. The ionome changes in mutant-inoculated plants was compared to WT-inoculated plants. Mutants inoculation changed the content of some elements in leaves differently than WT (Fig. 8). In water treatment (Fig. 8a), all the elements except Mn and Zn were significantly changed in *copB* mutant-inoculated plants. Among them, Ca, Fe, Mg and Na showed opposite tendencies of changes between *copB* mutant and WT inoculated plants. In *copA* mutant-inoculated plants, only Ca, Mg and Na were significantly different from WT inoculated plants, with Ca and Mg showing opposite tendencies. In 8 mM $CuSO_4$ treatment (Fig. 8b), most of the elements were not significantly changed between mutants and WT inoculated plants. Only K and Zn content in *copB* mutant-inoculated plants were decreased significantly compared with WT inoculated plants. From the heat map (Fig. 8c), ionome of Cu-amended plants were distinguished from non-Cu-amended plants. Meanwhile, mutants-inoculation clearly changed ionome of plants in water treatment. These changes were also present in Cu-amended treatment, but less intense than that of water treatment. *copB* mutant-inoculated plants with water treatment had similar ionome response pattern as Cu-amended plants, based on the Principal Component Analysis (PCA) results (Supplemental Fig. 2).

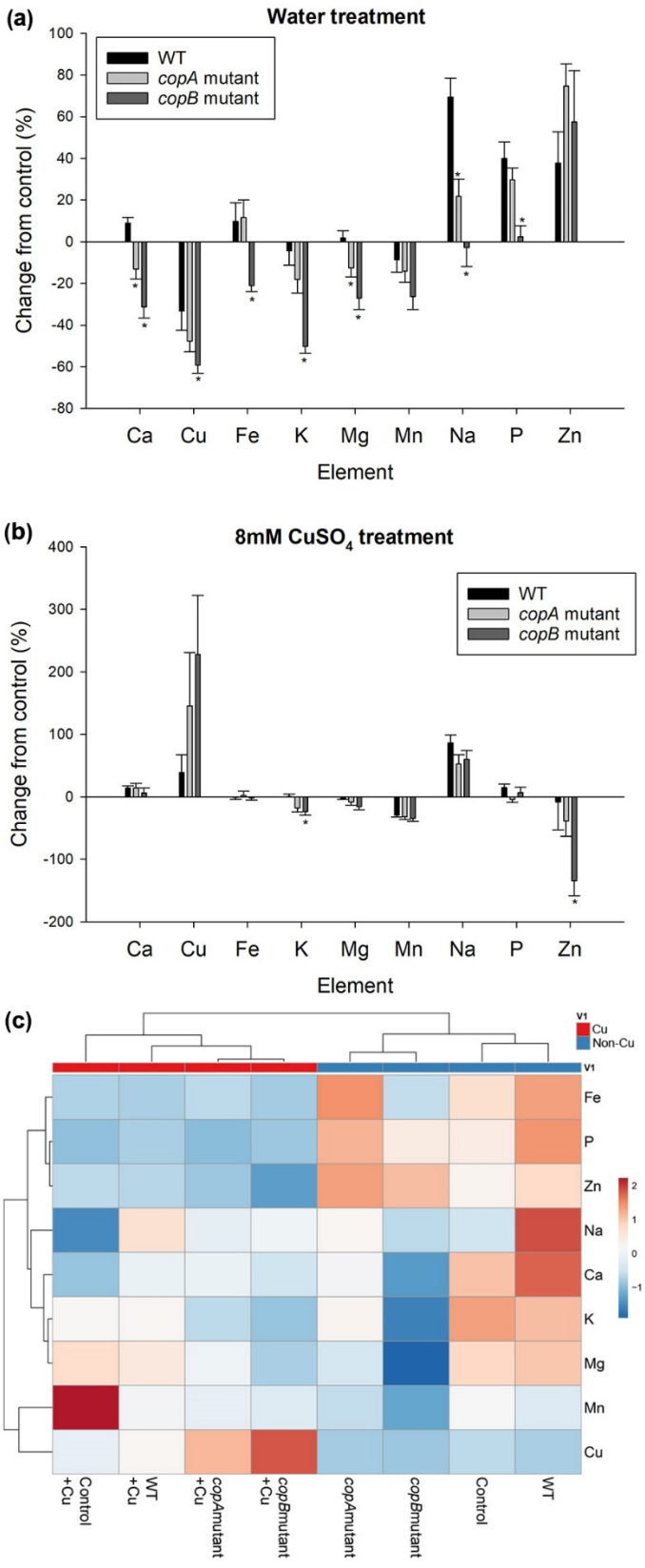
(a)
Water treatment
WT
copA mutant
copB mutant
Change from control (%)
100
80
60
40
20
0
-20
-40
-60
-80
Ca
Cu
Fe
K
Mg
Mn
Na
P
Zn
Element
(b)
8mM CuSO4 treatment
WT
copA mutant
copB mutant
Change from control (%)
400
300
200
100
0
-100
-200
Ca
Cu
Fe
K
Mg
Mn
Na
P
Zn
Element
(c)
V1
Cu
Non-Cu
V1
Fe
P
Zn
Na
Ca
K
Mg
Mn
Cu
2
1
0
-1
+Cu Control
+Cu WT
+Cu copAmutant
+Cu copBmutant
copAmutant
copBmutant
Control
WT

Fig. 8. Mutant and WT caused ionome changes in tobacco plant leaves

Ionome changes in leaves of plants treated with water ('Water treatment') (a) or 8mM $CuSO_4$ ('8mM $CuSO_4$ treatment') (b), and Heat map (c). Elements Ca, Cu, Fe, K, Mg, Mn, Na, P and Zn were measured form the same leaves samples used for Cu concentrations (Fig. 7). Plants were inoculated with WT, *copA* mutant or *copB* mutant. PBS inoculated plants were considered as control. Five leaves per plant and five plants per treatment were collected (n = 25/treatment). Values in graph represents means and error bars represent standard error of the mean. Data used in the graph corresponds to one representative experiment, and three independent experiments performed under the same conditions showed similar tendencies. *Indicates significant difference ($p < 0.05$) between mutants and WT according to Tukey's test (all pairwise multiple comparison procedures). Heat map was carried by ClustVis with default settings.

Discussion

In this study *copA* and *copB* mutants were analyzed under in vitro and in planta conditions at different Cu concentrations. Although both mutants were more sensitive in vitro to Cu than WT, they performed differently under different types of Cu treatments. Thus, *copA* and *copB* are believed to have different functions in Cu homoeostasis in *X. fastidiosa*. In planta experiments symptom development was more influenced by Cu in mutants than WT, indicating that Cu-handling is important for *X. fastidiosa* virulence in planta.

In the genome of *X. fastidiosa*, no homologues of the chromosome-encoded bacterial Cu resistance gene families (*cue* gene family and *cus* gene family) were found. However, a *cop* (copper resistance) gene family, which is homologous to *pco* (plasmid copper resistance) gene family in *E. coli* and is usually found in plant pathogenic bacteria is present. Unlike *pco* system in *E. coli* that is only found in plasmids, *cop* system can be found in plasmids or chromosomes.

In *P. syringae* pv. *tomato* (Ps), *pco* genes *copABCDRS* are localized within the plasmid (Cha & Cooksey, 1991). PsCopA functions as multicopper oxidases and can sequester Cu, and PsCopB is an outer membrane protein that is suggested to be important for exporting excess Cu (Cha & Cooksey, 1991, Cooksey, 1993). In *Xanthomonas* spp., *cop* system has three main components, *copLAB* that are present in all Cu resistant strains (Behlau et al., 2011). The location chromosomal or plasmid location of the *copAB* genes is correlated with role in Cu-homeostasis and resistance, respectively (Behlau, 2010). The *copA* and *copB* in *X. fastidiosa* studied here are homologous to *copA* and *copB* in *Xanthomonas* spp., with similarity of 72% for CopA and 47% for CopB at the protein level. CopA is a multicopper oxidase (MCO) in *Xanthomonas* spp. and given the high similarity to *X. fastidiosa* it will be expected to have similar function. This prediction is further supported by the secondary structure modelling of *X. fastidiosa* CopA via Phyre2 that indicates a high confident prediction of the multicopper oxidase fold (data not shown). The MCOs have multiple potential substrates including metals, siderophores, and different metabolic products. The loss of MCO in *Xanthomonas citri* pv. *citri* results in decreased Cu tolerance (Teixeira et al., 2008) and this is consistent with our observation in *X. fastidiosa*. Many of the experimental approaches used in the past to study CopA have been chronic treatments, where Cu is added throughout growth. The potential role of CopA to withstand Cu shock discovered here is novel. Meanwhile, the function of Cu sequestration found in *Pseudomonas* spp. was not observed in *X. fastidiosa copA*. The degree of Cu resistance that CopA and CopB are involved were also different among *Xanthomonas citri* pv. *citri* and *X. fastidiosa*. Although protein similarities were shared among these plant pathogenic bacteria, the specific functions of CopA and CopB might vary from bacteria to bacteria.

Gene expressions of *copA* and *copB* genes were both induced by low concentrations (0 to 250 μM) of chronic Cu treatments, and suppressed in high Cu concentration. These results are similar with former research in difference subspecies of *X. fastidiosa* (Rodrigues et al., 2008). We also noticed that the gene expressions were decreased at Cu deficiency condition (BCS treatment), which further confirm the importance of Cu in the regulation of these genes. Biofilm formation by *X. fastidiosa* is enhanced by addition of Cu at low concentration to the medium, while further increasing the Cu concentration eventually decreases biofilm formation (Cobine et al., 2013). Here we assessed biofilm formation of *copA* and *copB* mutants under variable Cu concentrations. The response curve to Cu was clearly shifted in the mutants, with enhanced biofilm formation at a lower concentration and inhibited at lower Cu concentrations than expected compared to WT. This suggests that Cu in these mutants is more available or accumulating to higher levels, which suggest lack of functional Cu exporter or loss of sequestration/detoxification. This would imply that if Cu is acting in a signaling role this would be enhanced in the mutants, triggering earlier biofilm formation that may affect virulence in planta.

One of the mechanisms that is related to Cu detoxification in gram-negative bacteria mentioned by Cooksey (1990) is Cu sequestration. Their study showed that when *Pseudomonas syringae* pv. *tomato* grew on Cu abundant media, its colony showed blue color as the result of Cu accumulation (Cha & Cooksey, 1991). Further study showed that CopA from *Pseudomonas syringae* pv. *tomato* could bind free Cu ions in the periplasm to sequester Cu. As a consequence, free Cu in cells and the damage it caused were decreased (Cha & Cooksey, 1991). In our study, we noticed significantly higher Cu accumulation in mutants under Cu added environments by ICP-OES measurements. However, until a lethal dose of Cu was added, no clear blue colonies or

blue cells were observed (data not shown). The overall high sensitivity to Cu in *X. fastidiosa* results in less Cu accumulation and could be the reason why there was no blue color observed. Motif sequences of 'His-X_{48}-Cys-X_4-His-X_4-Met' and 'Asp-His-X_2-Met-X_2-Met' was identified as possible Cu binding site in *Pseudomonas* sp. (Ouzounis & Sander, 1991, Cooksey, 1993). Based on protein sequence analysis, we found 6 repeats of 'Asp-His-X_2-Met' in *X. fastidiosa* CopB, that could be potential Cu-binding sites. These protein motifs were not found in *X. fastidiosa* CopA. The number of motifs in *X. fastidiosa* (6 in CopB and 0 in CopA) compared with *Pseudomonas syringae* pv. *tomato* (5 in CopB and 11 in CopA) may suggest lower resistance to Cu (Cha & Cooksey, 1991, Cooksey, 1993). Given the different lifestyle and environments of these two bacterial species, the levels of Cu exposure are markedly different. Further studies are required to confirm the Cu-binding functions for sites in CopB and CopA in *X. fastidiosa*. Sequestration prevents reactive Cu accumulation but the major mechanism to protect against this accumulation is Cu export. According to protein prediction, CopB could be a transmembrane protein, which could serve as a Cu exporter. In *copB* mutant, higher accumulation of Cu in cells is consistent with this prediction. Sequestration could have a role but does not appear to be the main mechanism of Cu detoxification in *X. fastidiosa*.

In acute Cu shock experiments, different responses of *copA* and *copB* mutants were observed. It is supposed that under acute Cu shock, the main way of Cu detoxification will be sequestration or oxidization, as opposed to Cu export. This is due to the saturation of the export systems that are normally poised for lower chronic Cu-concentrations. The dramatic toxicity of Cu shown by decreased viable cell populations of *copA* mutant under Cu shock suggests that this cell cannot detoxify Cu efficiently, even though total Cu is not elevated relative to the *copB* mutant which was more resistant (presumably due to the presence of CopA). The increased Cu accumulation in

the *copB* mutants may suggest it is functioning in the export process. The loss of oxidization of Cu(I) in bacterial cells leads to redox damage and mismetallation of important proteins including damage to Fe-S cluster enzymes. CopA acting as MCO could be facilitating oxidation of Cu(I) to less toxic Cu(II) to protect the cell. In *copB* mutant, we did see more Cu accumulated in cells, which may be due to less efficiency of Cu export, however acute cell death was prevented. This means when cells are facing high level of Cu toxicity in a short time, export is not the major method of detoxification. Therefore, we propose that oxidization and export of CopA and CopB work synergistically to deal with Cu stress, to complete the full function of Cu homeostasis in *X. fastidiosa*.

Ionomes of plants inoculated with the mutants were affected differently, especially in *copB* mutant (Fig. 8). It is possible that CopB being a putative Cu exporter may also influence other elements exportation and storage, especially divalent cations, such as Ca^{2+} and Mg^{2+}. The change in elemental composition of the pathogen could influence its host plant and affect plant ionome. In contrast, CopA as a possible Cu oxidase would have limited influence on other elements storage and export, and therefore more limited influence on plants ionome. All these changes were more pronounced in water treatments than Cu-amended treatments. A possible explanation is that Cu amendment in plants minimized the change brought by mutants' inoculation.

X. fastidiosa inside xylem vessels of plants are surrounded by a very defined, nutrient-poor environment. In our previous research, we noticed a trend of increasing symptoms when plants were treated with extra Cu. This phenomenon may be due to *X. fastidiosa* taking advantage of Cu homeostasis of plants and using the additional Cu to regulate virulence traits, such as biofilms (Ge et al., 2020). In this current study, we also noticed the increasing of symptoms in Cu homeostasis genes knock out mutants, and the influence of Cu was more obvious in mutants than

WT. In many bacterial animal pathogen, deletion of Cu related genes leads to Cu hypersensitivity and reduced in vivo virulence, such as *copA1* gene in *Pseudomonas aeruginosa* that is sensitive to high Cu concentration and significantly attenuates virulence in mice (Williams et al., 2020, Schwan et al., 2005). However, in some cases, mutation of Cu related genes lead to Cu hypersensitivity but not reduced virulence, such as Mmco multi-copper oxidase of *Mycobacterium tuberculosis* in mice (Ward et al., 2010, Ladomersky & Petris, 2015). Considering that the Cu concentration in sap was in a range that boosts growth and/or biofilm formation of *X. fastidiosa* (Ge et al., 2020, Cobine et al., 2013), this could explain the increase in disease symptoms. Under the same amount of extra Cu-amended, mutants would be faster to achieve the cytoplasmic amount of Cu they needed to boost biofilm formation, at lower concentration than that of WT. Plants inoculated with these mutants actually increased Cu concentration in plant sap and leaves. Clearly, Cu plays an important role during plant-pathogen interactions. Understanding Cu homeostasis in *X. fastidiosa* and how it influences pathogen in planta inoculation are significant towards disease control and management.

copA and c*opB* genes have been shown in this study to be necessary for full function of Cu homeostasis in *X. fastidiosa*. CopA and CopB were predicted to function differently as helping sequestration and export of Cu, respectively. The pathogen increased its virulence by efficiently managing plant Cu around its environment, taking the necessary Cu to facilitate its own growth and establishment. The ability of withstanding Cu stresses in *X. fastidiosa* could be an evolutionary adaptation to xylem vessels of plants, which have been exposed to years of Cu-containing microbicides amendments. Better understanding of how Cu influences pathogen-host interaction could help to adjust and accomplish disease management strategies.

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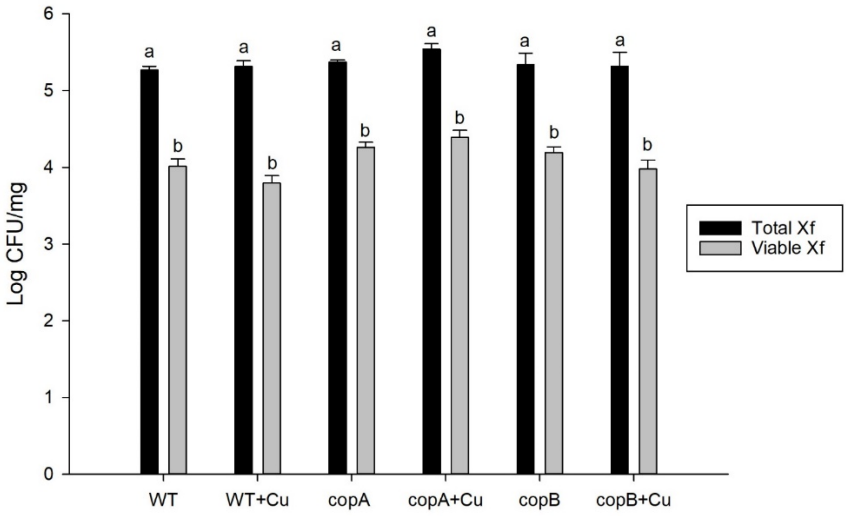
Supporting Information

Supplemental table 1. PCR primers used in this study

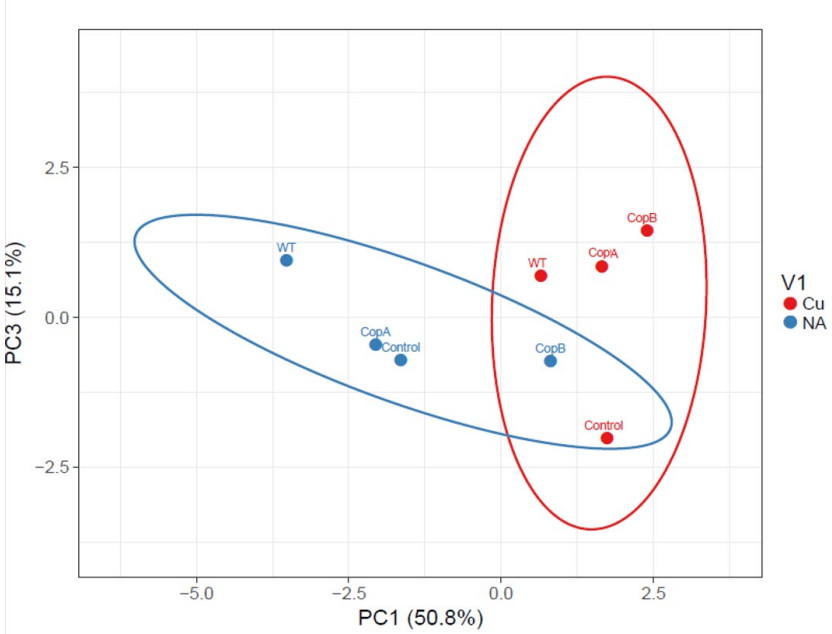
Primer	Function or target	Sequence	Source
Mutagenesis			
copAupF1	Upstream *copA*	CGATGCGAGACCTCTTCCTG	This study
copAupkanR1	Upstream *copA*	CAACACCTTCTTCACGAGGCAGAC GTCCGGCGCTTTGGCGACGGAACG	This study
copAdownF2	Downstream *copA*	GATTTTGAGACACAACGTGGCTTA TGAACACCCGTACCTGGTTCG	This study
copAkandown R1	Downstream *copA*	CTTGGAGGTTCGGTTGCAGGATC	This study
copBupF1	Upstream *copB*	CTGATTAATGGTTCGGCGATGAC	This study
copBUDR2	Upstream *copB*	GGTCAGGAACGTTCATGGGCGCAC CTGTACTTCACG	This study
copBDUF2	Downstream *copB*	CAGGTGCGCCCATGAACGTTCCTG ACCAGTGAAGCATC	This study
copBdownR3	Downstream *copB*	GTACGAATTTGCGTGGAACG	This study
KanF	Kanamycin cassette	GTCTGCCTCGTGAAGAAGGTGTTG	This study
KanR	Kanamycin cassette	AAGCCACGTTGTGTCTCAAAATC	This study
Confirmation of mutations			
copAF	*copA* gene deletion	ACCTATGATGTCCTGATCC	This study
copAR	*copA* gene deletion	CTGTACTTCACGCATCATG	This study
copBF	*copB* gene deletion	ATGAACACCCGTACCTGG	This study
copBR	*copB* gene deletion	TCAAAACCAGCAGCGTATC	This study
copAupF2	Kanamycin cassette replace	CATTGTGACTGCTTGCAG	This study
copAkanR	Kanamycin cassette replace	GATTCAGTCGTCACTCATG	This study
Gene expression in *X. fastidiosa*			
copAF2	Detection of *copA* gene	CAGGGCGTGAGATCGAATTA	This study
copAR2	Detection of *copA* gene	CACCGTAGTTGAGTCGTAAGG	This study
copBF2	Detection of *copB* gene	GTGCCGCGTTTCTGTATTTG	This study
copBR2	Detection of *copB* gene	CTGACTGAGTAGCAGGTCATATTT	This study
Quantification of *X. fastidiosa*			
HL5	Detection of *X. fastidiosa*	AAGGCAATAAACGCGCACTA	Francis et al. 2006
HL6	Detection of *X. fastidiosa*	GGTTTTGCTGACTGGCAACA	Francis et al. 2006
HLP	Detection of *X. fastidiosa*	FAM-TGGCAGGCAGCAACGATACGGCT-BHQ	Francis et al. 2006

Supplemental fig. 1. Quantification *X. fastidiosa* in tobacco plant.



Bacterial populations were assessed at the end of symptoms development. Total *X. fastidiosa* (Total Xf) population was quantified by qPCR, and viable *X. fastidiosa* (Viable Xf) was quantified by PMAxx-qPCR. *X. fastidiosa* WT ('WT'), *X. fastidiosa* WT with 8mM $CuSO_4$-amended treatment ('WT+Cu'); *copA* mutant ('copA') and *copA* mutant with 8mM $CuSO_4$-amended treatment ('copA+Cu'); *copB* mutant ('copB') and *copB* mutant with 8mM $CuSO_4$-amended treatment ('copB+Cu'). Different letters above bars indicate significant differences ($p < 0.05$) among all treatments according to Tukey's test (all pairwise multiple comparison procedures). Error bars correspond to standard error of the mean. Data used in the graphs corresponds to one representative experiment. Three independent experiments performed under the same conditions showed similar tendencies.

Supplemental fig. 2. Principal Component Analysis (PCA) of ionome of mutants-inoculated plants



PCA were carried by ClustVis with plants ionome data of *X. fastidiosa* WT ('WT'), *copA* mutant ('copA'), *copB* mutant ('copB'). Water treatment (NA) shown as "Blue" plots; 8mM $CuSO_4$-amended treatment (Cu) shown as "Red" plots.

Chapter 4: *cutC* gene in the xylem-limited pathogen *Xylella fastidiosa* is involved in Cu homeostasis/resistance and affects virulence

Abstract

Copper (Cu) is a crucial element for all living organisms to survive, however it can also be toxic. Plant pathogenic bacteria have been exposed to Cu stress for years, due to wide application of Cu-containing antimicrobials, leading to acquisition of Cu resistance mechanisms. Cu homeostasis/resistance mechanisms in *Xylella fastidiosa*, which is a Gram-negative, xylem-limited plant pathogenic bacterium that causes disease in many economically important crops worldwide, is poorly understood. One protein related to Cu tolerance in *Escherichia coli* and humans is CutC; a triosephosphate isomerase (TIM)-barrel protein that has a single Cu binding site and is thought to protect cells against accumulation of free Cu. Here we focused on analyzing *cutC* gene in *X. fastidiosa*, a homolog of *cutC* from *E. coli,* by site-directed mutagenesis. When compared with wild type (WT) *X. fastidiosa* strain TemeculaL, the *cutC* mutant showed increased sensitivity to Cu. The mutant showed decreased bacterial culturable populations (colony forming units, CFU) under short time exposure to high concentrations of Cu. When infecting plants in the greenhouse, *cutC* mutant showed decreased disease severity compared to WT, but when Cu was added to the watering solution, disease severity increased more in the mutant than the WT. We hypothesize that in *X. fastidiosa cutC* gene is involved in Cu resistance by binding Cu in cells, leading to Cu detoxification. The observation that CutC is important for *X. fastidiosa* virulence *in planta,* is probably related to the suggested role of Cu in biofilm formation. Unveiling the role of *cutC* gene in *X. fastidiosa* further confirms the important

role of Cu during this pathogen infection process and helps understanding Cu homeostasis/resistance in this and other plant/animal bacterial pathogens.

Introduction

Copper (Cu) resistance and homeostasis in prokaryotic and eukaryotis organisms have been studied for a long time, and it is still a very active area of research. Cu plays an essential role maintaining cellular physiology since it acts as a metal cofactor for many enzymes (Brown et al. 1992). However, overdose of Cu in cells leads to damage of cell membranes, proteins and DNA (Brown et al. 1992; Gupta et al. 1995; Kunjunni et al. 2016; Yoshida et al. 1993). It is crucial for living organisms to maintain homeostasis of intracellular Cu to survive in environments with changing Cu concentrations. In bacteria, Cu resistance and homeostasis usually relies on *cop*, *cus* and *cut* gene families (Brown et al. 1992; Cooksey 1990, 1993). Among them, the *cut* family has not been well studied, especially in plant pathogenic bacteria.

In *E. coli*, the *cut* family consist of six genes *cutABCDEF* that are related to Cu homeostasis and resistance, including Cu uptake and efflux, intracellular storage and delivery (Rouch et al. 1989). Mutation of one or more *cut* genes leads to increased Cu sensitivity. CutC is proposed to be a cytoplasmic Cu-binding protein, based on gene sequence analysis (Gupta et al. 1995; Rouch et al. 1989). Previous research found that a *cutC* mutant can accumulate Cu without changing Cu uptake kinetics (Gupta et al. 1995; Rouch et al. 1989). CutC is conserved among prokaryotic and eukaryotic organisms (Gupta et al. 1995; Li et al. 2010). Characterization has been conducted in humans and other eukaryotic organisms, in regard to protein structural features and physiological function. The human homolog of CutC (hCutC) has a TIM barrel structure and

binds one Cu+ per monomer via Cys31 and Cys52 (Li et al. 2010). *hCutC* silencing causes Cu-mediated metal stress in cells and leads to imbalanced distribution and metabolism of intracellular Cu, that eventually can induce cell apoptosis (Kunjunni et al. 2016). In *Enterococcus faecalis*, CutC partakes in the control of Cu homeostasis and is possibly related to Cu efflux (Latorre et al. 2011).

Xylella fastidiosa is a gram-negative bacteria pathogen that could infect many plant hosts (Hopkins and Purcell 2002; Purcell and Hopkins 1996). In the U.S., common diseases caused by *X. fastidiosa* includes Pierce's disease of grapevine and bacterial leaf scorch of blueberry (Oliver et al. 2015). In other parts of the world, it causes citrus variegated chlorosis mainly in South America and olive quick decline syndrome in Europe (Chang et al. 1993; Janse and Obradovic 2010; Martelli et al. 2016; Saponari et al. 2017). *X. fastidiosa*-caused diseases have a huge economic impact worldwide (Janse and Obradovic 2010; Schneider et al. 2020; Tumber et al. 2014), therefore understanding the surviving mechanisms of *X. fastidiosa* under complex environments will help finding ways to control this pathogen. Studies on the molecular basis of Cu homeostasis and resistance in *X. fastidiosa* are limited (Chanper 3), and there are no previous studies regarding *cut* genes family. Rodrigues et al. (2008) have pointed out the increase of *cutC* transcriptional level when *X. fastidiosa* were grown under increased Cu concentrations. But there has been no functional characterization of this gene in plant pathogens. In this research, we constructed a *cutC* knockout mutant by site-specific mutagenesis and studied *cutC* gene function in *X. fastidiosa* as well as its effects on *X. fastidiosa* virulence *in planta*. This is the first time *cutC* was functionally characterized in plant pathogenic bacteria.

Materials and Methods

Bacterial strains and culture conditions.

Xylella fastidiosa subsp. *fastidiosa* type strain TemeculaL was used as the wild type (WT) strain in this study. *X. fastidiosa* WT and mutants were incubated at 28℃. Media used for culturing *X. fastidiosa* strains were Periwinkle Wilt (PW) agar plates (Davis et al. 1981), Pierce's Disease 2 (PD2) broth (Davis et al. 1980) and PD3 medium (Davis et al. 1981), with or without kanamycin (Km) (50 μg/ml). Bacterial suspensions of *X. fastidiosa* were prepared as follows. *X. fastidiosa* strains were streaked from -80 ℃ frozen glycerol (20%) stocks on PW agar plates (Km was used for mutants) for 5-7 days, and then re-streaked onto new PW plates for additional 5-7 days. For plant inoculation experiments bacterial cultures from PW agar plates were scraped with sterilized loops and suspended in PBS (phosphate-buffered saline). Final concentration of bacterial suspensions used for plant inoculations was set at OD_{600nm}=0.8. For in vitro experiments, bacterial suspensions were prepared similarly as mentioned above, but with PD2 media instead of PBS.

Gene expression of *cutC*

Gene expression of *cutC* in WT *X. fastidiosa* was assessed under Cu-replete (100 μM, 200 μM, 250 μM and 350 μM $CuSO_4$) and Cu-depleted (150 μM BCS: bathocuproine sulfate) conditions. WT *X. fastidiosa* cells were prepared as mentioned above and grown in 5ml PD2 media with initial OD_{600nm}=0.01. Bacteria were cultured for 7 days at 28 ℃ with shaking at 140 rpm. After

that incubation period, bacteria were collected by 3 min 13,000x g centrifugation. Quick-RNA MiniPrep kit (Zymo Research, Irvine, CA) was used to extract total RNA. DNase I (Thermo Scientific, Pittsburgh, PA) treatment was carried as needed to remove contaminant DNA in samples. Single-strand cDNA was synthesized with 1 µg RNA of each sample by reverse transcription using the Maxima First Strand cDNA synthesis kit for reverse transcription-quantitative PCR (RT-qPCR) (Thermo Scientific, Pittsburgh, PA). cDNA samples from each treatment were diluted 5 times with RNA/DNA free water. Primers used for *cutC* were designed using PrimerQuest software (Integrated DNA Technologies): cutCF2 (5'AGTTGTGTCATGGG TTGGAG3') and cutCR2 (5'GAGGCCGGATCAACACATAAA3'). Internal control used in this research was *gyrB* gene, as previously described (Cruz et al. 2014). The qPCR reactions consisted of incubation at 95°C for 1 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min, carried by ABI 7500 real-time PCR system (Applied Biosystems, Foster City, CA) with SYBR green (Bio-Rad, Hercules, CA). The fold change in gene expression compared with WT was calculated according to the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001).

Construction of mutant strain.

In this study *X. fastidiosa cutC* (PD0585) null mutants were generated by natural competence and homologous recombination, according to a previously-described protocol (Kandel et al. 2018). Specifically, *X. fastidiosa* WT genomic DNA was extracted by using a modified CTAB protocol (Doyle and Doyle 1987). Then, ~1000bp upstream and downstream of *cutC* open reading frames (ORF PD0586) were amplified by PCR with two primer pairs cutCupF1 (5'GCG GGGTCTTCTTTGCTCGGATTG3') and cutCupkanR1 (5'CTGGTATGAGTCAGCAACACC

TTACCCGCCTCCCATGATCCTCGTG3'), cutCdownkanF3 (5'CAATGTAACATCAGAGAT TTTGAGTCTGTTGGGCAGATGGTTCTG3') and cutCdownR3 (5'CTTTCCACAATCGCAT CAACATC3'). The cutCupkanR1 and cutCdownkanF3 primers have ~21 bp overlapping sequences with Km cassette sequences for overlap extension PCR. A Km cassette was amplified from pUC4K with primer pair kanF (5'AAGGTGTTGCTGACTCATACCAG3') and kanR (5'CAATGTAACATCAGAGATTTTGAGT3'). PCR products were collected and purified with Gel purification kit (Zymo Research, Irvine, CA). Three PCR fragments: gene upstream, downstream and Km cassette were used as templates for overlap extension PCR. Primer pair cutCupF1/cutCdownR3 were used to get one fusion PCR fragment. After that, the purified fusion PCR fragments were mixed with 10-µl OD_{600nm}=0.25 bacterial suspension of WT strain on PD3 agar plates. After 3 days of co-culture at 28 ℃, bacterial cells were suspended in 200 µl PD3 broth. Twenty- and 100 µl bacterial suspension were plated onto PW+Km plates for selection. After ~2 weeks, single colonies were picked and re-streaked to new PW+Km plates. Colonies confirmation was performed with PCR, and further confirmation was done by sequencing. PCR was performed with a standard protocol, using an iProof High-Fidelity PCR kit (Bio-Rad, Hercules, CA) in an S1000 thermal cycler (Bio-Rad, Hercules, CA).

Characterize mutant growth under different Cu concentrations

Growth curves of *X. fastidiosa* WT and *cutC* knockout mutant were performed using PD2 media or PD2 with 150µM BCS, using OD_{600nm} values to assess bacteria growth over time. WT and mutant were sub-streaked from glycerol stocks and bacterial suspensions were prepared as mentioned above. Bacteria was grown in 50 mL falcon tubes with 5 mL media (PD2 media or

PD2 media with 150µM BCS) (initial OD_{600nm} ~ 0.01). Bacterial suspensions were mixed by vortex before OD_{600nm} measurements recorded over seven days for growth curve analyses. Three tubes from each treatment were used for each OD_{600nm} measurement, and three independent experiments were conducted.

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

Biofilm formation and planktonic growth of *X. fastidiosa* WT and mutants were quantified in 96-well plates following a previously described method (Chen et al. 2017; Cruz et al. 2012), with some modifications. Briefly, bacteria were grown in 96-wells plates with initial OD_{600nm}= 0.01, using PD2 broth alone or amended with 4 different concentrations of $CuSO_4$ (50 µM, 100 µM, 150 µM and 200 µM). 200 µl of media were used on each well, 8 repetitions (wells) per treatment were considered for each treatment, and 3 plates were used for each independent experiment (n=24). 96-wells plates were shaken at 140 rpm for 5 days at 28 ℃. After 5 days, 150 µl of supernatant from the bacterial culture were removed to a new 96-wells plates, and OD_{600nm} measurements were used to calculate planktonic growth. The original culture plates were rinsed two times with Milli-Q water. Then, 230 µl of 0.1% crystal violet were added to each well. After 20 min, crystal violet was removed, and plates were rinsed 3 times with Milli-Q water. Finally, 230 µl 95% ethanol were added to plates and incubated for 5 to 10 mins. OD_{600nm} values were used to calculate biofilm formation. Three independent experiment were carried for this assessment.

Mutant population quantification under Cu shock

X. fastidiosa WT and mutant were grown and prepared as mentioned above. Both strains were cultured in 5ml PD2 media with initial OD_{600nm} ~ 0.01. After 3 days of culturing at 28 ℃ with 140 rpm shaking conditions, $CuSO_4$ were added to a final concentration of 500 μM as a Cu shock treatment, while same volume of PD2 media were added to culture as control treatment. After 12h of Cu shock treatment, bacterial cells were collected by 4℃ centrifugation at 4,000x g for 10 min. Then cells were re-suspended in 1 ml PD2 media and diluted to bacterial suspension of 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6} dilutions. Ten μl of bacterial dilutions were plated to PW plates. For each strain and each bacterial dilution, 8 repetitions were prepared. After ~ 2 weeks of culturing at 28 ℃, bacteria population of each strains with Cu shock treatment and control treatment were calculated by CFU counting. Three independent experiment were performed.

Quantification of twitching motility

Twitching motility of *X. fastidiosa* was assessed by the fringe width of colony when grown in PW plates. *cutC* mutant and WT strain were grown under different $CuSO_4$ (50 μM, 100 μM, 150 μM and 200 μM) supplemented conditions for their twitching motility assessment. All PW plates using in twitching motility assessment were prepared without BSA amendment. Each strain was grown in PW plates from -80 ℃ frozen glycerol (20%) stocks for 5-7 days, and then re-streaked onto new plates for additional 5-7 days. After that, bacterial cells were collected by sterilized loops and spotted onto new PW plates. *cutC* mutant and WT were spotted in the same plates with 4 spots for each strain. Three plates were used for each treatment. After two days of culture at 28 ℃, spots were observed under Nikon Eclipse Ti inverted microscope (Nikon, Melville, NY) (40x

objective). NIS-Elements Advanced Research 3.01(Nikon, Melville, NY) was used to quantify the width of spot's peripheral fringe. Each spot was measured three times.

Greenhouse experiments

X. fastidiosa WT and *cutC* mutant strains virulence was compared as previously described (De La Fuente et al. 2013) by using the model plant tobacco (*Nicotiana tabacum* 'Petite Havana SR1', plant introduction number 552516). Tobacco plants were grown and prepared following previously described methods (De La Fuente et al. 2013; Francis et al. 2008; Ge et al. 2020). Briefly, 6-week-old tobacco plants were prepared by trimming off upper leaves and keeping only the three healthy lower leaves . Bacterial inoculum (OD_{600nm}=0.8) was prepared as described above. Small wounds were punctured at the base of the leaf petioles by 23-gauge needles. For plants to take up bacterial inoculum, 20 µl of bacteria suspension were added to the small wounds. For control group, plants were similarly treated with PBS buffer only. After the first inoculation, one new shoot was kept for later growth and the rest of the shoots were trimmed. Second inoculation was carried at 1 week after the first one following the same protocol. For each bacterial strain treatment, half of the plants (9 plants) were treated with 200 mL 8 mM $CuSO_4$, and the other half (9 plants) with tap water. Treatments with additional Cu started one day after the first inoculation and were repeated once a week.

Disease symptom calculation

Symptom of disease started to show ~75-85 days after the first inoculation. The number of plants showing symptoms was recorded as disease incidence. For disease severity two measurements were used: i) Leaf scorch scale: leaf scorch symptoms on each leaf were given scores (0-5) according to the following scale: leaves showing 100% of its surface area with scorch symptoms were ranked as 5; leaves with 75% of scorch were ranked as 4; leaves with 50% scorched area as 3; leaves with 25% scorched area as 2, and if only the tip part of leaves were scorched, they were considered as 1. No symptoms were considered as 0. All the leaf score symptoms were added for each plant and the resulting number was considered as the score for disease severity of each plant; ii) Percentage of scorched leaves: the number of leaves showing scorch symptoms per plant was recorded (leaf scorch % = (#scorched leaves*100)/#total leaves). Data was recorded weekly after the first symptoms appeared. The area under the disease progress curve (AUDPC) was calculated based on the midpoint rule method (Campbell and Madden 1990) as follows: $AUDPC= \Sigma [(yi + yi+1)/2](ti+1 – ti)$, where i = the number of assessment times, y = disease severity score for each plant at each assessment, and t = time at each assessment.

Ionome Characterization

Cu concentration in bacterial cells was measured by Inductively Coupled Plasma with Optical Emission Spectroscopy (ICP-OES, Perkin Elmer 7100 DV, Waltham, MA) as previously described (Cobine et al. 2013). Bacteria were grown in PD2 with either 0 µM, 20 µM or 50 µM $CuSO_4$ (initial OD_{600nm}=0.01). After 7 days of incubation with shaking at 140 rpm (28 °C), bacterial cells were collected in 1.5 ml tubes by centrifugation at 10,000x g for 3 min. Then, bacteria were washed with Milli-Q water twice. For each sample, 100 µl of mineral-free

concentrated nitric acid (OPTIMA, Fisher Scientific) were added for digestion and treated for one hour at 100 ℃. Later, each sample was diluted with 200 µl Milli-Q water and ready to be analyzed.

For leaf ionome characterization, 5 plants were selected from each treatment and 5 leaves above the inoculation point of each selected plant were collected. Ionome characterization was carried by ICP-OES (Perkin Elmer 7100 DV, Waltham, MA) following a previously-described protocol with some modifications (De La Fuente et al. 2013). Briefly, leaves were collected and dried at 80 ℃ for 1 hour. Totally dried leaves were ground to fine powder with a mortar and pestle. Then, 10 mg of fine leaves powder were taken from each sample and digested in 200 µl of mineral-free concentrated nitric acid (OPTIMA, Fisher Scientific) for 1 hour at 100℃. After that, samples were diluted by adding 800 µl Milli-Q water. Remaining particulates in samples were removed by centrifugation (13,000x g for 5 min). Samples were analyzed by ICP-OES with simultaneous measurement of Ca, Fe, Cu, Zn, Mn, Mg, K, Na, and P. Mineral element concentrations were determined by comparing emission intensities to standard curves created from certified standards (SPEXCertiprep, Metuchen, NJ). Standard curves were confirmed by reanalysis of standard solutions diluted in a matrix equivalent to the sample. Individual readings (average of two intensity measurements) and repeated analysis of individual samples, showed less than 5% variation (data not shown).

For sap ionome characterization, similar protocol as leaves ionome characterization was used. From each treatment, sap of 5 plants were collected using a Scholander pump model 600 pressure chamber (PMS Instrument Company, Corvallis, OR) as described previously (Oliver et al. 2014). Sap samples were diluted two times with Milli-Q water, and then directly used for ICP-OES analysis.

Total and viable bacterial population quantification

Quantitative polymerase chain reaction (qPCR) and PMAxx (Biotium, Hayward, CA, USA) were used to quantify total and viable population of *X. fastidiosa* strains in plant, respectively, as previously described (De La Fuente et al. 2013; Ge et al. 2020). Briefly, fresh petioles plants from each treatment were cut into small pieces. Around 100 mg of fresh tissues were weight and ground 2-3 min by using a mini Beadbeater 96 (Biospec Products, Bartlesville, OK) with 2.0 mm beads (ZIRCONIA, Biospec Products, Bartlesville, OK) at the highest speed. After grounded, samples were suspended in 1.5 ml PD2 broth. From there, 500 µl-samples were used for total bacteria population quantification. A second 500-µl sample was treated with PMAxx for viable bacteria population quantification. PMAxx with a final concentration of 50 µM was added to each sample and incubated in the dark for 5 min. Right after that, samples were exposed to a strong white light (700W halogen portable work light, Husky Co., Atlanta, GA) for 10 min. Samples were placed on ice to prevent overheating during the light treatment. DNA of total and viable bacteria samples were extract by a modified CTAB protocol (Doyle and Doyle 1987). Quantification of *X. fastidiosa* populations were carried by qPCR following a previously described protocol (De La Fuente et al. 2013) with primer pair HL5 (5'AAGGCAATAAACG CGCACTA3') and HL6 (5'GGTTTTGCTGACTGGCAACA3'), and probe HLP (5'FAM-TGGCAGGCAGCAACGATACGGCT-BHQ3').

Statistical analyses

For gene expression, biofilm formation, twitching motility, culturable bacteria population changes and disease symptoms, statistical analyses were done using the two-tailed Student's *t-test* (p <0.050) (Microsoft Excel® 2016 for Windows). For the analysis of Cu concentrations in bacterial cell, plant sap and leaf, leaf ionomes, and bacterial population quantification all pairwise multiple comparison procedures were used with Tukey's test (Kruskal Wallis for non-normal data) using SigmaPlot 14.0 (Systat Software, Inc. SigmaPlot for Windows) (p <0.050).

Results

cutC gene expression is influenced by Cu and it does not influence *X. fastidiosa* growth *in vitro*.

Gene expression of *cutC* in *X. fastidiosa* WT was assesed under different Cu concentrations (Fig. 1). Expression of *cutC* gene was significantly increased when Cu concentrations were increased to 200 and 250 µM. When *X. fastidiosa* WT cells were grown under 100 µM, 350 µM Cu and deficiency condition (150 µM BCS), expression of *cutC* gene was not significantly changed from the control (PD2 media).

The *cutC* knockout mutant was constructed using natural competence of *X. fastidiosa* as shown in previous publications (Chen et al. 2017; Kandel et al. 2018). The absence of the *cutC* gene on the knockout mutant and the insertion of a Km-resistance cassette were confirmed by PCR. Further confirmation was carried out by sequencing to assess that upstream and downstream sequences of *cutC* genes were not modified by the insertion (data not shown).

The growth of *cutC* mutant in PD2 media was assessed in this research. The results showed that *cutC* mutant shared the same growth pattern with WT when they grew under PD2 media (Fig. 2). In day 5, significant lower growth was noticed in *cutC* mutant. However, the differences between *cutC* mutant and WT were non-significant the rest of the days assessed, therefore it was considered that both strains have the same growth pattern. The growth of *cutC* mutant under Cu deficiency conditions (150 µM BCS) was also assessed, and there was non-significant differences between *cutC* mutant and WT.

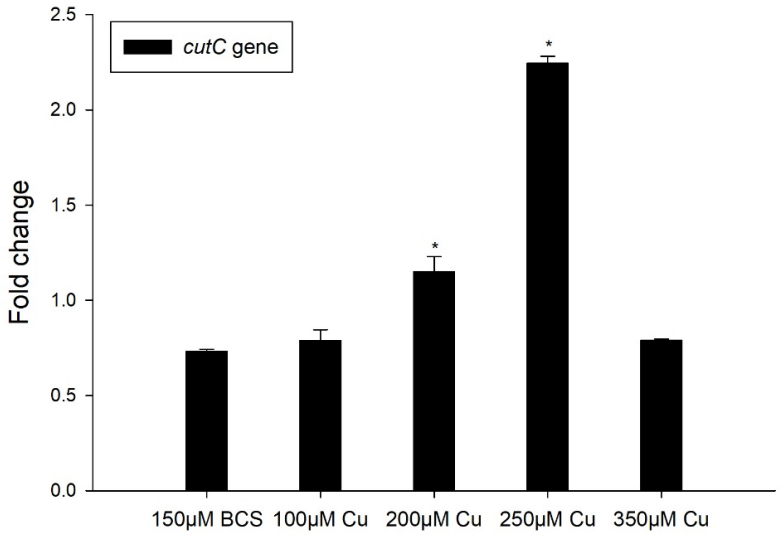


Fig. 1. Gene expression of *cutC* gene

X. fastidiosa WT cells were grown in test tubes for 7 days in PD2 media amended with different concentrations of $CuSO_4$ and BCS (Cu chelator). Gene expression was measured by RT-qPCR, using *gyrB* as internal control. Fold change of gene expresion was calculated by comparing to that of unamended PD2 media. Mean values of 3 independent repetitions (n= 9) are shown in graph, and error bars represent standard error of the mean. * indicate significant differences in gene expression as determined by the two-tailed Student's *t-test*.

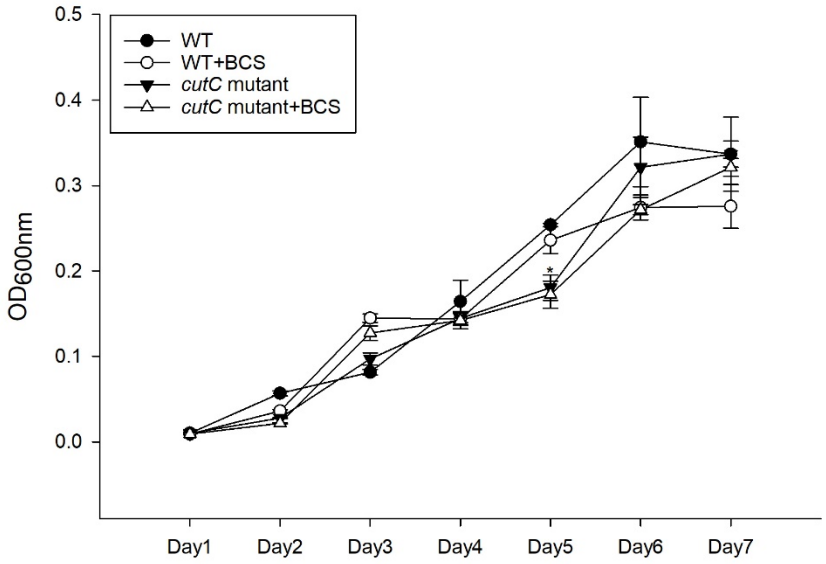


Fig. 2. Growth curve of WT and *cutC* mutant in PD2 and BCS

Mutants and WT were grown in 50mL falcon tubes for 7 days with 5mL PD2 media or 150 µM BCS amended PD2 media. OD_{600nm} value was mearsured every 24h. Mean values of 3 repetitions are shown in graph, and error bars represent standard error of the mean. Three individual experiments were carried on with the same results. * indicate significant differences OD_{600nm} value between mutant and WT as determined by the two-tailed Student's *t-test*.

cutC gene does not influence _X. fastidiosa_ biofilm formation under chronic Cu treatments.

The *cutC* mutant was grown under chronic Cu treatments (50 µM, 100 µM, 150 µM and 250 µM $CuSO_4$) for biofilm formation and planktonic growth assessments. The results showed that biofilm formation of *cutC* mutant increased at 100 µM and 150 µM $CuSO_4$-amended conditions, while decreased at 250 µM $CuSO_4$-amended condition. The biofilm formation of WT increased

at 50 µM, 100 µM and 150 µM $CuSO_4$-amended conditions, while decreased at 250 µM $CuSO_4$-amended condition. The pattern of WT biofilm formation was consisted with previous publications (Cobine et al. 2013). The only difference between the mutant and WT was at 50 µM $CuSO_4$-amended condition, when WT increased biofilm formation while *cutC* mutant did not. However, this difference was non-significant ($p = 0.291$). Biofilm formation of the two strains was considered as following the same tendencies under chronic Cu treatments. The planktonic growth of both *cutC* mutant and WT was not significantly influenced by Cu-amended treatments.

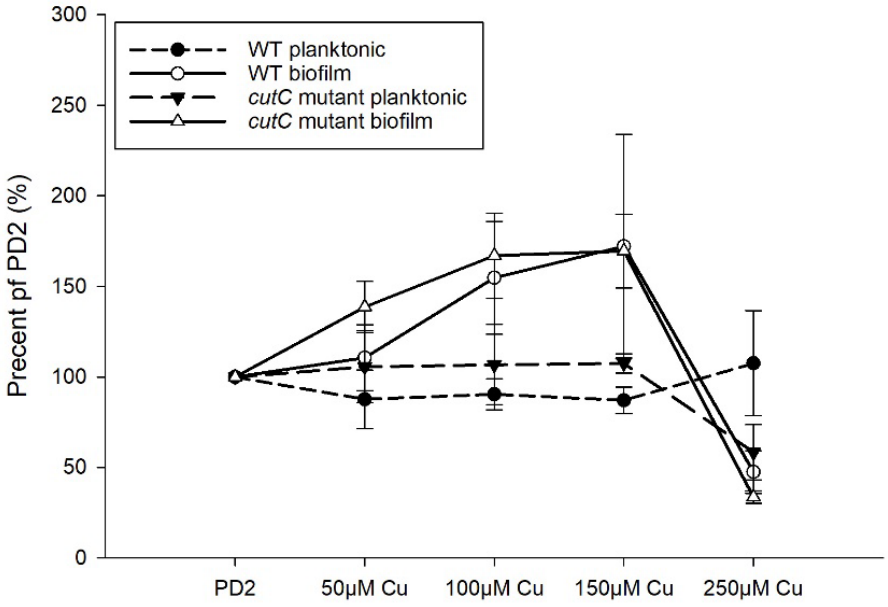


Fig. 3. Biofilm formation and planktonic growth by WT and *cutC* mutant

Mutant and WT cells were grown in 96-wells plates for 5 days with PD2 media amended with different concentrations of $CuSO_4$. Biofilm was assessed by OD_{600} of crystal violet staining. The value of PD2 100% represents the growth of bacteria in PD2 media without any amendments, and was used as control. Numbers above 100% represent growth increase, and below 100% represent growth inhibition. No significant difference were found between the mutant and WT as determined by the two-tailed Student's *t-test*.

***cutC* gene is important for *X. fastidiosa* to withstand Cu shock.**

Cu shock was experimentally set as 500µM $CuSO_4$ treatment for 12h. Culturable bacteria populations of WT and *cutC* mutant were assessed after exposure to Cu shock. The change of culturable bacteria populations as compared to strains growing in non-amended media were recorded as different times (Table 1). WT culturable population (measured as CFU on agar plates) decreased ~6 times after Cu shock. On the other hand, populations of *cutC* mutant under the same Cu treatment markedly decreased by ~50 times, rendering significantly lower populations than WT ($p < 0.05$). Differences between WT and mutant after Cu shock treatment was easily noticed by growth in agar plates (Fig. 4). It was concluded that *cutC* gene has an important role in *X. fastidiosa* withstanding Cu shock.

CFU/ml	Control	500µM Cu	Times decreased
WT	$2.7x10^8$	$4.3x10^7$	6.3X
***cutC* mutant**	$2.3x10^8$	$4.8x10^6$	48X*

Table 1. Culturable bacterial population (CFU) changes after Cu shock.

WT and mutant cells were grown in PD2 liquid media for 3 days, then acute Cu treatment (500µM for 12h) and mock treatment were added. Culturable bacteria was quantified as CFU by plating in PW agar plates after Cu treatment and mock treatment (control). * indicate significant differences in CFU reduction between mutants and WT as determined by two-tailed Student's *t-test*.

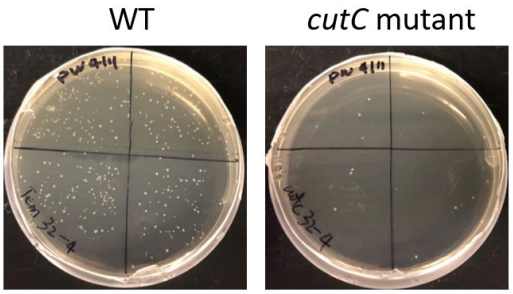


Fig. 4. Culturable populations on agar plates after Cu shock

WT and mutant cells in Cu shock treatment and control were collected and diluted for plating. Here the plates of WT and mutant after Cu shock treatment were showed with 10^4 times dilution of original cell suspension.

cutC mutant has limited influence on twitching motility

Twitching motility is an important virulence factor for *X. fastidiosa* that mainly help the pathogen move against the sap flow. Here twitching motility of *X. fastidiosa* was assessed by the colony fringe width on agar plates. WT and *cutC* mutant were grown in PW plates and in PW plates amended with 4 different concentration of $CuSO_4$ (50 μM, 100 μM, 150 μM and 250 μM). The measurement of colonies fringe width showed that both WT and the mutant increased its twitching motility when Cu concentration increased from PW (0 μM) to 100 μM, and decreased at 150 μM and 250 μM. Compared with WT, *cutC* mutant had wider fringe at 100 μM $CuSO_4$ and shorter fringe at 150 μM $CuSO_4$. Both of them were statistically significant ($p < 0.05$), yet, the differences between the mutant and WT were small. In other Cu concentrations, the fringe width of *cutC* mutant does not significantly differentiated with WT. *cutC* gene had very limited influence on *X. fastidiosa* twitching motility.

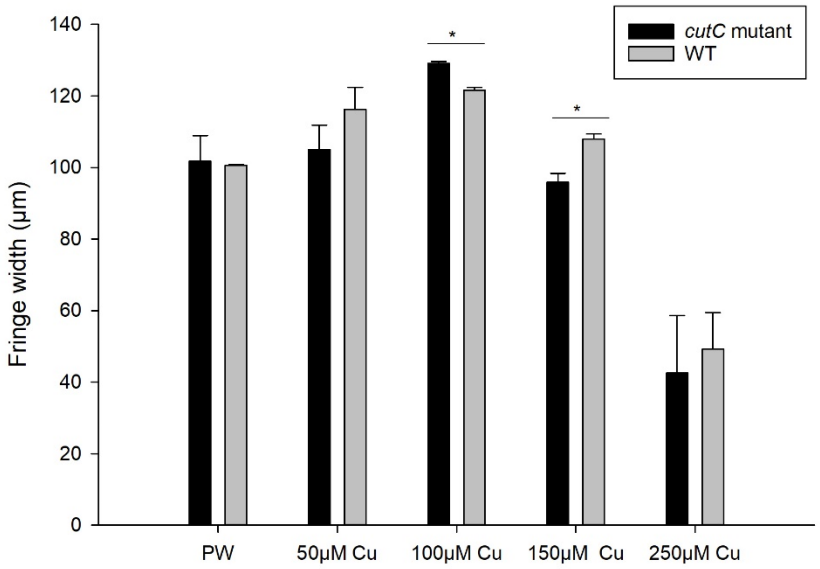


Fig. 5. Twitching motility of *cutC* mutant

Twitching motility of *cutC* mutant and WT were assessed by the colony fringe width. Colony of each strain were grown in PW plates or PW with 4 different concentration of $CuSO_4$. Each treatment had 12 colonies for each strain, and each colony was used for 4 measurements. Mean values of 48 repetitions are shown in graph, and error bars represent standard error of the mean. * indicate significant differences ($p < 0.05$) between WT and *cutC* mutant in each treatment according to by two-tailed Student's *t-test*.

cutC mutation increased intracellular Cu accumulation

To investigate how does *cutC* gene influence Cu accumulation in *X. fastidiosa*. Cu contents of *cutC* mutant and WT were measured by ICP-OES when they grew in PD2 and PD2 with Cu supplemented (20 µM and 50 µM $CuSO_4$) conditions. The results pointed out that *cutC* mutant had significantly ($p < 0.05$) higher Cu accumulation than WT under 50 µM $CuSO_4$ condition.

When cultured in PD2 media and 20 µM $CuSO_4$ conditions, *cutC* mutant did not show significant differences with WT ($p = 0.827$ and $p = 0.998$, respectively).

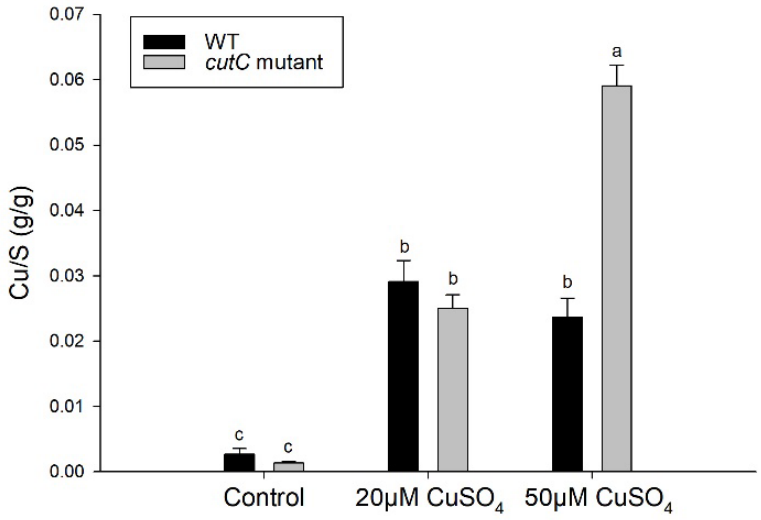


Fig. 6. Cu accumulation in *cutC* mutants.

Mutant and WT were grown for 7 days in test tubes with PD2 media amended with 20µM or 50µM $CuSO_4$. Cu content in cells was measured by ICP-OES. Mean values are shown in graph, and error bars represent standard error of the mean. Data used in the graph corresponds to one representative experiment, and three independent experiments performed under the same conditions showed similar tendencies. Different letters above bars indicate significant differences ($p < 0.05$) among all treatments according to one-way ANOVA .

cutC mutation does not influence plant Cu accumulation.

cutC mutant and WT were inoculated in the model plant tobacco (*Nicotiana tabacum* Petite Havana SR1) to examine if its inoculation could change Cu accumulation *in planta* differently than the WT. Cu concentration in plant sap and leaves was measured by ICP-OES as previously

described (De La Fuente et al. 2013; Ge et al. 2020). The results showed Cu content in sap and leaves for all 8mM $CuSO_4$-amended treated plants were significantly ($p < 0.05$) increased compared with water-treated plants. This result is consistent with previous research of our group (Ge et al. 2020). The difference of Cu content in sap and leaves between *cutC* mutant and WT were non statistically significant (sap: $p = 0.398$; leaves: $p = 0.594$). In sap, Cu concentrations of control, WT and *cutC* mutant were ~ 60-70 µM, with no statistical significance between them. In leaves, Cu concentrations of control, WT and *cutC* mutant were 35±5 mg/kg, 45±9 mg/kg and 53±7 mg/kg, respectively. *cutC* mutant had the highest Cu content in leaves among three groups, however, this result was non-statistically significant.

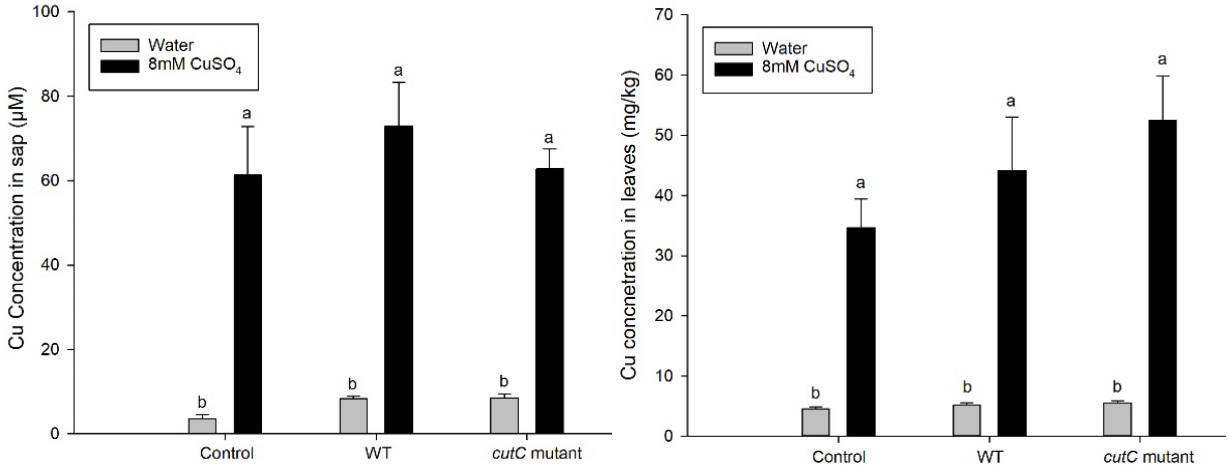


Fig. 7. Cu concentration in sap (left) and leaf (right) of tobacco plants

Sap and leaves samples were collected from tobacco plants grown in the greenhouse (n = 5/treatment). Plants were inoculated with PBS ('Control'), *X. fastidiosa* WT ('WT') or *X. fastidiosa cutC* knockout mutant ('*cutC* mutant'). Plants were watered either with 200ml of 8mM $CuSO_4$ or tap water. Mean values are shown in graph, and error bars represent standard error of the mean. Data used in the graph corresponds to one representative experiment, and three independent experiments performed under the same conditions showed similar tendencies.

Different letters above bars indicate significant differences ($p < 0.05$) among all treatments according to Tukey's test (all pairwise multiple comparison procedures).

cutC mutant is less virulent than WT in planta but induce severe symptom under $CuSO_4$-amended treatment.

cutC mutant virulence _in planta_ was assessed in comparison to WT. 8mM $CuSO_4$-amended treatment was applied once per week to assess how Cu influence _cutC_ mutant _in planta_ virulence. After 75-85 days post-inoculation, depending on the season when the experiment was conducted, disease symptoms of leaf scorch started to show. Once the initial symptoms were observed, disease incidence and severity from WT and mutant inoculated plants were assessed (Fig. 8). Disease incidence results showed that _cutC_ mutant had less incidence than WT in both water and Cu-amended treatments. Although 8mM $CuSO_4$-amended treatment increased _cutC_ mutant incidence from 50% to 80%, the number was still lower than the incidence of WT that was 100% under both treatments (Fig. 8A). _cutC_ mutant inoculated plants had significantly higher disease severity (score of leaf scorch, Fig. 8B) in 8mM $CuSO_4$-amended treatment compared with water treatment in week 3, 4 and 5. However, for WT inoculated plants no statistical difference was found between the treatments. Considering severity measured as the percentage of scorched leaves out of total leaves, _cutC_ mutant had significantly lower percentage (~20% lower) than WT in water treatment (Fig. 8C). However, in 8mM $CuSO_4$-amended treatment, WT and _cutC_ mutant had the same percentage of scorched leaves towards the last weeks of assessment (Fig. 8C). _cutC_ mutant inoculated plants showed decrease of disease incidence and severity under water treatment. The assessment of area under the disease progress curve (AUDPC) showed significantly increased AUDPC of _cutC_ mutant inoculated plants

treated with 8mM $CuSO_4$ compared with water treatment (Fig. 8D). The 8mM $CuSO_4$-amended treatment lead to increase of disease symptoms by all the parameters measured here. Meanwhile, the leaf scorch score distribution in plant were measured by calculate the percentage of scorch score of bottom leaves, middle leaves and top leaves takes out of the plant leaves total score, respectively (Fig. 8E). The results showed that there was no statistical differences among the groups, but a tendency of less symptoms at top leaves in plants infected by *cutC* mutant compared with that of WT. This indicates *cutC* mutant might cause uneven symptom distribution in plants. However, WT caused more even symptom distribution in plants.

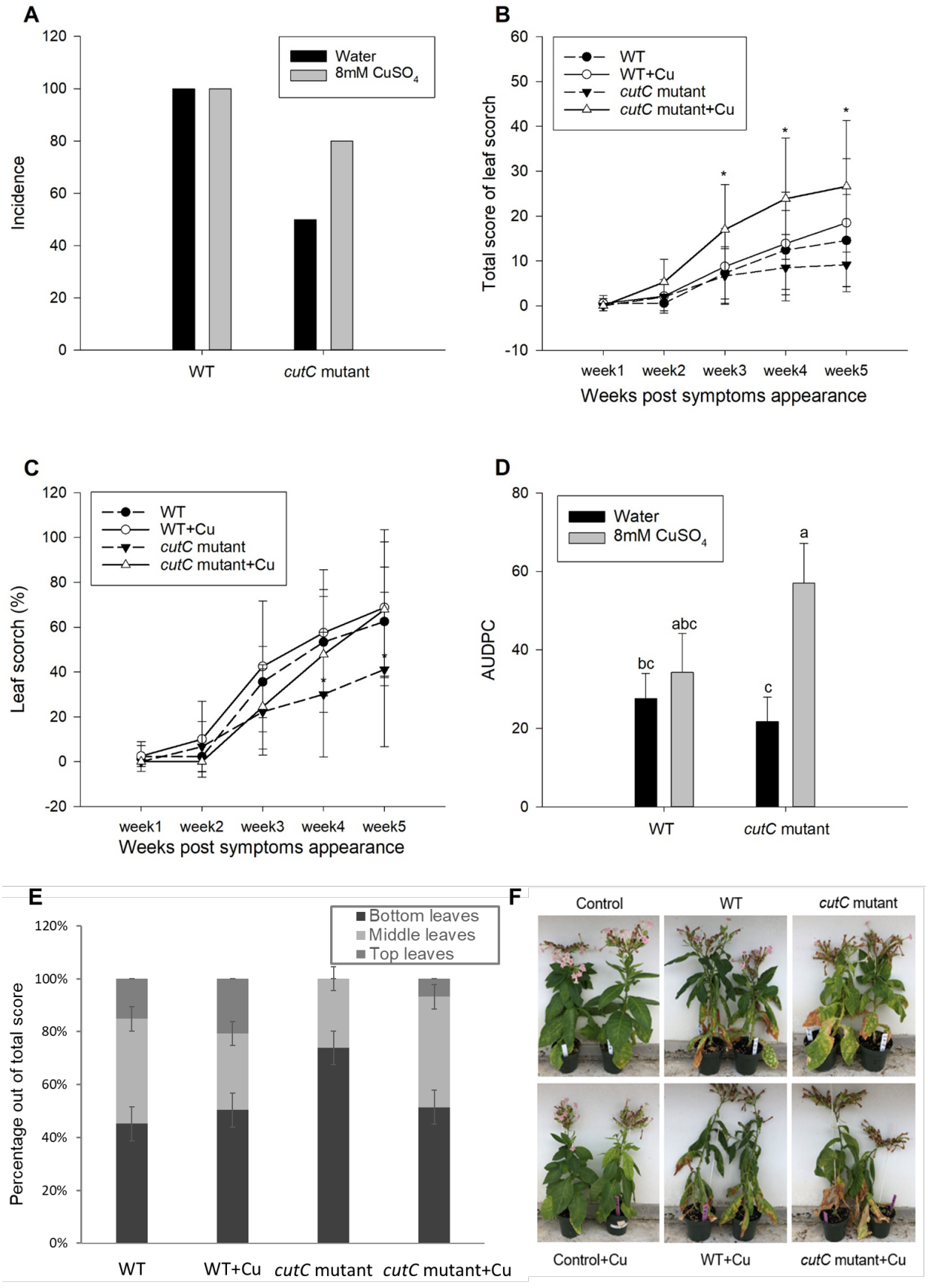

A
Incidence
Water
8mM CuSO4
WT
cutC mutant
B
Total score of leaf scorch
WT
WT+Cu
cutC mutant
cutC mutant+Cu
week1
week2
week3
week4
week5
Weeks post symptoms appearance
C
Leaf scorch (%)
WT
WT+Cu
cutC mutant
cutC mutant+Cu
Weeks post symptoms appearance
D
AUDPC
Water
8mM CuSO4
bc
abc
c
a
WT
cutC mutant
E
Percentage out of total score
Bottom leaves
Middle leaves
Top leaves
WT
WT+Cu
cutC mutant
cutC mutant+Cu
F
Control
WT
cutC mutant
Control+Cu
WT+Cu
cutC mutant+Cu

Fig. 8. Symptom development in tobacco plants by WT and *cutC* mutant under $CuSO_4$-amended treatments.

Disease incidence(A), severity (B and C), AUDPC (D), leaf scorch score distribution (E) and symptom of plants(F) were measured for plants infected with WT and *cutC* mutant. (A) Incidence: #plants with symptom/# total plants (B) Disease severity: Total score of leaf scorch in each plant. (C) Disease severity: Percentage of scorched leaves: the number of leaves showing scorch symptoms per plant was recorded (leaf scorch % = (#scorched leaves*100)/#total leaves). (D) AUDPC= Σ [(yi + yi+1)/2](ti+1 – ti), where i = the number of assessment times, y = total score of leaf scorch in each plant at each assessment, and t = time at each assessment. (E) leaf scorch score distribution: Percentage of leaf scorch score in different positions out of total leaf scorch score. (F) Plant pictures with leaf scorch symptom were record at week 5. Eight plants were considered for each treatment. Error bars correspond to standard error of the means. Data used for graph corresponds to one representative experiment. Three independent experiments performed under the same conditions showed similar tendencies. * in B indicate significant differences ($p < 0.05$) between treatmens in same strain and * in C indicate significant differences ($p < 0.05$) between mutant and WT in same teatment as determined by two-tailed Student's *t-test*. Different letters above bars in D indicate significant differences ($p < 0.05$) among all treatments according to two-tailed Student's *t-test*.

cutC mutant populations does not change under Cu-amended treatment _in planta_

The total and viable populations of WT and *cutC* mutant in inoculated plants were assessed by qPCR and PMAxx-qPCR, respectively. The results showed no significant change in bacterial populations in *cutC* mutant with and without $CuSO_4$-amended treatments compared to WT. Total bacteria populations of mutants and WT were all between 5 to 5.5 log CFU/mg. The viable bacteria populations were ~4 log CFU/mg. These results were consistent with our previous

results of Cu related genes knockout mutants in planta bacterial populations (Ge et al. 2020)(Chapter 3).

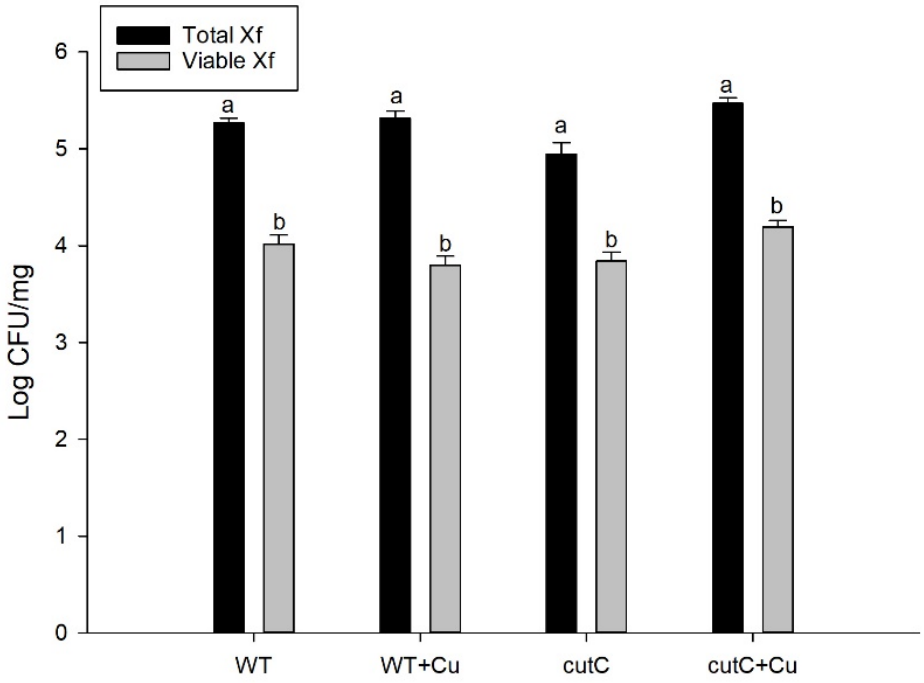


Fig. 9. Quantification of total and viable *X. fastidiosa* populations in tobacco plants.

Bacterial populations were assessed at the end of symptoms development. Total *X. fastidiosa* (Total Xf) population was quantified by qPCR, and viable *X. fastidiosa* (Viable Xf) was quantified by PMAxx-qPCR. *X. fastidiosa* WT ('WT'), *X. fastidiosa* WT with 8mM $CuSO_4$-amended treatment ('WT+Cu'), *cutC* mutant ('cutC') and *cutC* mutant with 8mM $CuSO_4$-amended treatment ('cutC+Cu'). Error bars correspond to standard error of the mean. Data used in the graphs corresponds to one representative experiment. Three independent experiments performed under the same conditions showed similar tendencies. Different letters above bars indicate significant differences ($p < 0.05$) among all treatments according to Tukey's test (all pairwise multiple comparison procedures).

Discussion

In this study, we functionally characterized a *cutC* gene knockout mutant in *X. fastidiosa*, constituting the first characterization of such gene family in plant pathogenic bacteria. The *cutC* gene knockout mutant was characterized in regard to growth and virulence under different Cu stresses *in vitro* and *in planta*. We conclude that *cutC* gene is important for *X. fastidiosa* to withstand Cu shock and is involved in virulence in plants.

When growth and biofilm formation of *cutC* gene knockout mutant were assessed under chronic Cu treatments, no significant differences were found between the mutant and WT. In *E. coli*, it has been reported that *cutC* gene more likely works under high Cu concentration as a complement system for Cu resistance (Brown et al. 1992; Gupta et al. 1995; Li et al. 2010). The chronic Cu conditions that was tested involve relatively low Cu concentrations; this could explain the reason why the growth of *cutC* mutant was not influenced by chronic Cu treatments. When under Cu shock, a treatment of high Cu concentration for short time, deletion of *cutC* gene did cause significant decrease of culturable bacteria population in *X. fastidiosa*. This result proved that in *X. fastidiosa cutC* gene was also function as handling the response to high, sudden Cu concentration, just as in *E. coli*. Just as the *copA* mutant (chapter 3) that have been studied before, *cutC* mutant played an important role in *X. fastidiosa* withstanding Cu shock. *cutC* mutant culturable bacteria population decreased ~50 times, which was significantly higher than WT growth reduction (~6 times) under Cu shock, but less intense than that of *copA* mutant, which decreased ~110 times (chapter 3). Here we hypotheses that *cutC* gene and *copA* gene may function in the same pathway or are correlated with each other in the function of withstanding Cu shock. If these two genes participate in two different pathways, knocking out one gene may not cause much influence on *X. fastidiosa* due to functional redundancy. *cutC* and *copA* gene were

believed to have direct or indirect connection. Based on the Cu resistance model proposed by Brown et al. (1992), *pcoA* and *cutC* both work towards Cu export in *E. coli*. Since *copA* and *cutC* genes in *X. fastidiosa* are homologs to *pcoA* and *cutC* in *E. coli,* respectively, they may share the same role in *X. fastidiosa* that is withstanding Cu shock and may not be replaceable by each other. Nevertheless, on our results indicated the mutation of *cutC* gene in *X. fastidiosa* caused significanlty increased of intracellular Cu content when it grew under Cu supplimented condition. This find is consiste with the results came out by Gupta's group in 1995 that *cutC* gene lead to intracellular accumulation of Cu in *E. coli*. They further suggested CutC functioning as a cytoplasmic Cu-binding protein in an efflux pathway for Cu (Gupta et al. 1995). Similar results and conclusions were noticed in the research of *Enterococcus faecalis* (Latorre et al. 2011). However, in the research of *hCutC* in HepG2 cells, total cellular Cu does not changed. This make it less likely that hCutC participate in cellular Cu export (Kunjunni et al. 2016). Although not always consist in different organisms, *cutC*-induced intracellular Cu accumulation was noticed, which make CutC protien highly possible participated in Cu efflux process. Therefore, it is highly possible that knocking out the *cutC* gene in *X. fastidiosa* created an imbalance in Cu homeostasis inside the cell resulting in Cu sensitivity. Therefore, CutC protein is more likely help defend *X. fastidiosa* against Cu toxicity by serving as Cu-binding protein in a Cu efflux pathway, leading to Cu detoxification. *cutC* mutant *in planta* virulence assessment showed that it caused low incidence and severity in water treatment, compared to WT. However, deletion of *cutC* gene in *X. fastidiosa* led to severe symptoms in infected plants when Cu-amended treatment was applied. This was different from that in WT, the disease development and symptom distributed relative evenly in each plants and leaves. Although Cu amendment leads to the trend of increasing disease severity in WT, it is less intense than that of *cutC* mutant.

qPCR results showed that *cutC* mutant bacteria populations in plant were not different than that of WT. This means different distributions of symptom in *cutC* mutant and WT inoculated plants were not related to their total or viable populations. The change of other virulence factors, such as twitching, attachment and biofilm formation, could be the possible reasons. One hypothesis was that symptom distribution of *cutC* mutant may be caused by the changing of its twitching ability. Previous research showed that *cutC* gene was upregulated under Ca amended environment, and Ca could influence *X. fastidiosa* twitching motility (Parker et al. 2016). However, when the twitching motility of *cutC* mutant was assessed by in vitro experiment, there was practically non-significant differences between *cutC* mutant and WT. The reduced symptoms in the acropetal regions caused by *cutC* mutant compared to WT, it is probably caused by differences in downstream movement of the bacteria, and not related to twitching. The interaction of pathogen and plants need to be considered in this situation.

X. fastidiosa CutC protein shares 46% identity of 3D structural model template with human CutC protein (hCutC). The study of hCutC protein structure provides clues for *X. fastidiosa* CutC protein function and structural characteristics. The study of hCutC biochemical and structural reveal that it can bind Cu(I) with an stoichiometry of 1:1 and the potential Cu(I)-binding sites were located in the inner surface of C-terminal end of TIM-barrel consist with two Cys residues (Li et al. 2010). However, we did not found any possible Cu binding sites in *X. fastidiosa* CutC protein sequence analyses (data not shown), according to two possible Cu(I) binding amino acids repeat sequences that were mentioned in previous publications: 'His-X_{48}-Cys-X_4-His-X_4-Met'; 'Asp-His-X_2-Met-X_2-Met' or 'Met-X_4-Met-X_2-Met' (Cooksey 1993; Odermatt et al. 1993; Ouzounis and Sander 1991). Cu binding motif are not always consistent in different organisms, and not all Cu-binding motifs/ligands are known (Li et al. 2010; Zhang and Gladyshev 2010).

Thus, failure of finding the above-mentioned repeat sequences does not mean Cu binding site were missing in CutC protein. Protein study of *X. fastidiosa* CutC is needed to better clarify this question. The ability of Cu binding in CutC protein could explain why *cutC* gene is more important for to withstand Cu shock than chronic Cu treatments.

In conclusion, our results indicate that *cutC* gene has a role in Cu resistance and virulence of *X. fastidiosa*. This gene is necessary for *X. fastidiosa* to withstand Cu shock, but less important for resisting chronic Cu treatments. The absence of *cutC* gene influences accumulation of cellular Cu. We hypothesize that *cutC* gene is more likely functioning as Cu-binding protein to facilitate the process of Cu efflux, resulting in Cu detoxification especially under Cu shock. In addition, deletion of *cutC* gene in the pathogen lower its virulence in plant under normal condition but increases its virulence under Cu-amended condition. The functional study of *cutC* gene in *X. fastidiosa* helps to improve our understanding of Cu resistance mechanisms in this pathogen (Chapter 3). Interestingly, we showed here that Cu resistance of this plant pathogen is closely related with virulence, therefore will have an impact in disease management.

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Chapter 5: Phenotypic and genetic characterization of Cu homeostasis/resistance in strains of *Xylella fastidiosa* isolated in the US

Abstract

Xylella fastidiosa is a gram-negative bacterial pathogen that causes severe diseases and asymptomatic colonization in more than 600 different crops and plants worldwide. This bacterium has not only a broad host range but also a wide geographical distribution. Previous research suggested that *X. fastidiosa* isolates from different geographical locations and host species have different features related with their virulence. Cu homeostasis/resistance of *X. fastidiosa* strains from different geographical locations and host species have not been characterized. Here, phylogeny of Cu-related genes (*copA*, *copB*, *copL* and *cutC*) among 74 genomes of *X. fastidiosa* was analyzed. The phylogenetic relationships among these genes basically followed *X. fastidiosa* subspecies classifications. Meanwhile, minimum inhibitory concentration of Cu (Cu MIC) of *X. fastidiosa* strains was assessed in 55 strains including 43 whose genome sequences were available. Relationships among Cu MIC, phylogenetic relationships and host and location of isolation were assessed. However, no correlation was found between these characteristics. Further analysis focused on a group of *X. fastidiosa* isolates from Georgia's vineyards. In general, higher cellular Cu, lower Cu MIC and increment of biofilm/total cells ratio were noticed in these isolates under Cu amendments compared with the control type strain originally isolated from California (TemeculaL). Based on our preliminary data, we cannot conclude if Cu resistance or homeostasis in *X. fastidiosa* is related with host and/or location of isolation. Further research is needed to better understand this question and to provide novel ideas for pathogen control and disease management.

Introduction

As mentioned in previous chapters, *X. fastidiosa* can colonizes and infects around 600 different host species, including agricultural crops, landscape trees and weeds (EFSA 2020; Hopkins and Purcell 2002). Meanwhile, *X. fastidiosa* has a wide geographical distribution; it has been reported in many countries and regions, such as the U.S., Brazil, Italy, Spain, and elsewhere (Almeida et al. 2008; Cariddi et al. 2014; Hopkins 1989; Olmo et al. 2017). Researchers showed *X. fastidiosa* that isolated from different geography locations and host species have differences in their virulence and infection symptomatology (Almeida et al. 2008; Almeida and Purcell 2003; Parker et al. 2012). Meanwhile, a study of *X. fastidiosa* isolates from different locations in California pointed out the importance of environmental factors on the pathogen's adaptation (Vanhove et al. 2020). Cu, a commonly used element in agriculture for disease control, could be an important factor in the environment that shapes the pathogen's virulence, principally by affecting its resistance mechanisms (Teixeira et al. 2008). Research in *Xanthomonas* spp. isolated from 12 different countries pointed out that Cu resistant strains were not distributed evenly in these locations (Behlau et al. 2013). Cu resistance developed in some strains of *Xanthomonas* spp. related with frequent sprays of Cu-based bactericides as well as horizontal transfer of Cu resistance genes (Behlau et al. 2013; Graham and Gottwald 1991). Cu resistance among isolates of the plant pathogen *X. fastidiosa* strains have not been studied yet.

These bring out an interesting question, whether there is a correlation between Cu resistant ability of these isolates and their geographical location, host species or genetical relationships. In this chapter, we wanted to investigate this by assessing the Cu MIC of *X. fastidiosa* strains, and discussing the relationship between their geographical location, host species, as well as

phylogeny of four Cu-related genes. We further discussed these questions in a small scale with a group of *X. fastidiosa* strains isolated form Georgia's vineyards.

Materials and Methods

Phylogenetic analysis

Phylogenetic trees were constructed using Molecular Evolutionary Genetics Analysis version 7.0 (MEGA7) software. Whole genomes of each isolate and strain were obtained either from sequences results of our lab or Almeida's lab (unpublished data), or from the database of National Center for Biotechnology Information (NCBI). Then, the target gene sequences for each isolate were selected by blasting whole genomes with corresponding genes from Temecula1 by using Basic Local Alignment Search Tool for Nucleotides (BLASTN). After that, partial nucleotide sequences of Cu resistance gene *copL* (PD0099), *copA* (PD0100), *copB* (PD0101) and *cutC* (PD0586) were aligned by default settings of MUSCLE in MAGA7. The alignments were assembled into a maximum-likelihood tree with 1000 bootstraps. The bootstrap values, as percentage out of 1000 replicates that the associated strains were clustered together in the bootstrap test, are shown next to the branches (supplemental figure 1). Branches with bootstrap values below 70% were collapsed. Phylogenetic trees were further adjusted in Phylo.io (www. Phylo.io) to make it easier to view (Robinson et al. 2016). Branches were collapsed when all the strains in a cluster belong to a same subspecies.

Positive selection analysis

To check if *copL*, *copA*, *copB* and *cutC* genes were under positive selection, analysis were carried in Datamonkey adaptive evolution serve (www. Datamonkey.org) (Delport et al. 2010). Alignment of each gene was obtained by using the default settings of MUSCLE in MAGA7. Stop codons in the alignment of each gene were removed following the software instructions (Poon et al. 2009). Alignments were upload to the websites as .meg format. Positive selection of the genes were analyzed by the fixed effects likelihood (FEL) method with default settings. Positive selection for each codon was considered as non-synonymous substitution (dN) > random synonymous substitution (dS) (dN/dS > 1); while negative selection was when dN < dS (dN/dS < 1)(Poon et al. 2009).

Cu MIC measurements

Cu MIC measurements were performed following protocols described in previous publications (Naranjo et al. 2020; Peterson and Shanholtzer 1992), with some modifications. Bacterial isolates and strains were streaked onto PW agar plates from -80 ℃ frozen glycerol (20%) stocks. After 5-7 days of first streaking, bacteria were re-streaked onto a new PW agar plates for another 5-7 days. After the second streaking, these bacterial cells were ready to use for later experiment. Bacterial cells were scraped from PW agar plates and suspended into PD2 liquid media. The optical density (OD_{600nm}) of bacterial suspensions were adjusted to 0.2 for later use. 190 µL PD2 media or PD2 media amended with different concentrations of $CuSO_4$ (0 µM, 150 µM, 200 µM, 250 µM, 300 µM, 350 µM, 400 µM) and 10 µL bacterial suspensions (OD_{600nm}=0.2) were added to each well of 96-wells plate. The final concentration of bacteria in each well was OD_{600nm}=

0.01. Bacteria were cultured with 140 rpm shaking condition at 28 ℃. After 7 days of culturing, 150 µl of supernatant of bacterial culture was removed to a new 96-wells plate, and OD_{600nm} was measured for planktonic growth calculations. For biofilm growth measurements, original plates were rinsed with Milli-Q water for 2 times to remove planktonic cells. Then, 230 µl of 0.1% crystal violet were added to each well, incubated at room temperature for 20 min with frequently shaking. After that, crystal violet was removed carefully from each well with pipets. Plates were rinsed with Milli-Q water for 2 to 3 times. Finally, each well was filled with 230 µl 95% ethanol and incubated for 5 to 10 min. OD_{600nm} values were measured to calculate biofilm formation. All the procedures of liquid adding and removing were performed under gentle and carefully pipetting, to minimize the loss of biofilm cells. The minimal Cu concentration that significantly ($p < 0.05$) inhibit bacteria total growth (total growth = planktonic growth + biofilm growth) was selected as Cu MIC.

Cu accumulation in cells

Cu content in bacterial cells after Cu-amended treatments were measured by Inductively Coupled Plasma with Optical Emission Spectroscopy (ICP-OES, Perkin Elmer 7100 DV, Waltham, MA) as previously described (Cobine et al. 2013; Ge et al. 2020), with some modifications. Bacteria were grown in 5mL PD2 media or PD2 media amended with 50 µM $CuSO_4$, with initial OD_{600nm} equal to 0.01. Bacterial cells were collected after 7 days of incubation. Bacterial cells were washed twice in Milli-Q water to remove media. In each tube, 100 µl of mineral-free concentrated nitric acid (OPTIMA, Fisher Scientific) were added in each sample for digestion. The digestion was processed under 100 ℃ heat treatment for one hour. Later, samples were

diluted with 200 µl Milli-Q water. The analysis was carried by ICP-OES with simultaneous measurement of Ca, Fe, Cu, Zn, Mn, S, Mg, K, Na, and P. Concentrations of mineral elements in each sample were determined by comparing emission intensities to certified standards curves (SPEXCertiprep, Metuchen, NJ), which confirmed by reanalysis of standard solutions diluted in a matrix equivalent to the sample. Two repetitions of individual readings (each individual reading is average of two intensity measurements) showed less than 5% variation.

Biofilm/total cells ratio

Biofilm/total cells ratio was calculated using the sulfur (S) concentration obtained by ICP-OES as follows: S concentration in biofilm cells/S concentration total cells. Bacteria were prepared and cultured as described above for measurements of Cu accumulation. After 7 days of culturing under PD2 media or PD2 media amended with 50 µM $CuSO_4$, biofilm and planktonic cells were carefully separated with pipetting and collected by centrifugation. Cells were washed with Milli-Q water to remove media and measured by ICP-OES under the same conditions. The S content in cells is linearly correlated with the cell number, and was used as an indicator of cell numbers (Cobine et al. 2013). Therefore, here biofilm/total cells ratio was represented by S content in biofilm cells/ total cells.

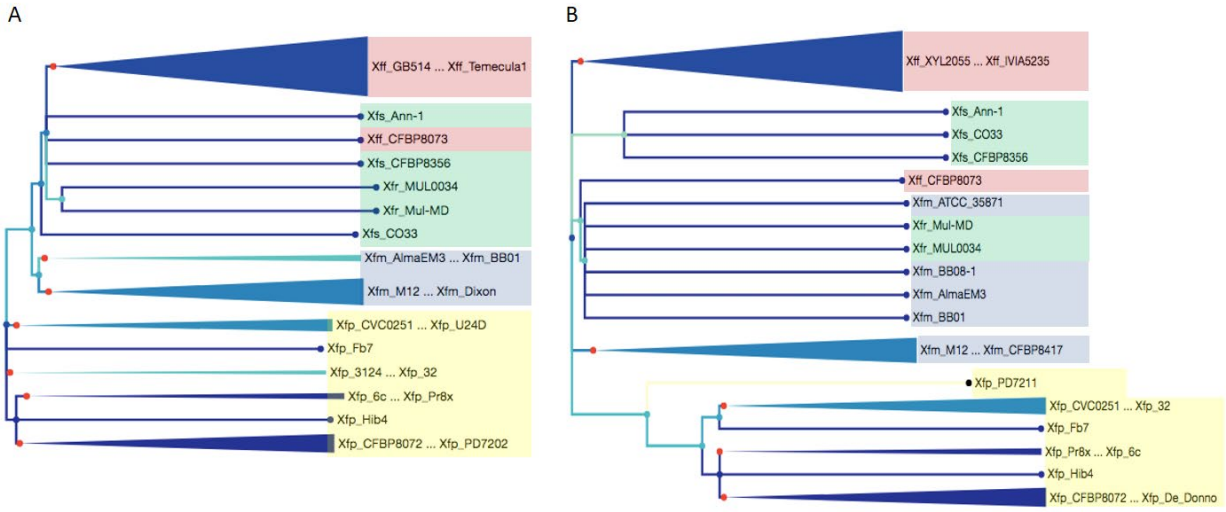
Results and Discussion

Phylogenetic analysis of Cu-related genes *copA*, *copB*, *copL* and *cutC*

To understand how Cu-related genes *copA*, *copB*, *copL* and *cutC* evolved among *X. fastidiosa* strains, phylogenetic analysis of the four Cu-related genes were carried in this study. The genomes of 74 *X. fastidiosa* strains were download from NCBI. The Cu-related genes of each strains were extracted by nucleotide blast with the genes from type strain Temecula1 though BLASTN in NCBI. Phylogenetic trees were made by using Maximum-likelihood method in MEGA7 and modified in phylo.io (Robinson et al. 2016) (Fig. 1). The strains were named according to subspecies classification as follows: Xff: *X. fastidiosa* subsp. *fastidiosa*; Xfm: *X. fastidiosa* subsp. *multiplex*; Xfs: *X. fastidiosa* subsp. *sandyi*; Xfr: *X. fastidiosa* subsp. *morus*; Xfp: *X. fastidiosa* subsp. *pauca*.

Overall, high DNA sequence identity of the Cu resistance genes were noticed among the strains with above 95% for *copA*, *copL* and *cutC*; above 90% for *copB*. Phylogenetic analysis highlighted the slight differences among the sequences of these genes among strains and separated them in different groups. From the results, *copL* was shown to be more conserved among the strains as it had least divergences, as well as *cutC* that was also highly conserved among the four genes. *copA* and *copB* were less conserved compared with the other two. These genes phylogenetical relationships followed the *X. fastidiosa* subspecies classification with few exceptions. Similarly, high identity (>90%) of Cu resistance genes (*copL*, *copA* and *copB*) were also noticed in *Xanthomonas* spp. (Behlau et al. 2013). However, in that case the phylogenetic analysis of Cu resistant strains revealed a geographic-based grouping pattern instead of species-based grouping pattern (Behlau et al. 2013).

Similar grouping and cluster patterns of phylogenetic trees were found between *copA* and *copB*, *copL* and *cutC*. In *copL* and *cutC* phylogenetic trees, the strains from Xfs, Xfm and Xfr were mixed with the Xff. However, the genes from these strains were closely related with Xff but separated in different clusters in *copA* and *copB* phylogenetic trees. Interestingly, an exception was the *copL* gene of Xfs strain CFBP8356 that was closely related to Xfp instead of the other Xfs (Fig. 1C); the other three genes analyzed of this strain were grouped with Xfs. Overall, *copL* as a regulator of *copAB* operon evolved differently compared to *copA* and *copB* genes. *copL* being more conserved among *X. fastidiosa* strains may be an indication of the tight homeostatic regulation of Cu across populations of this pathogen.



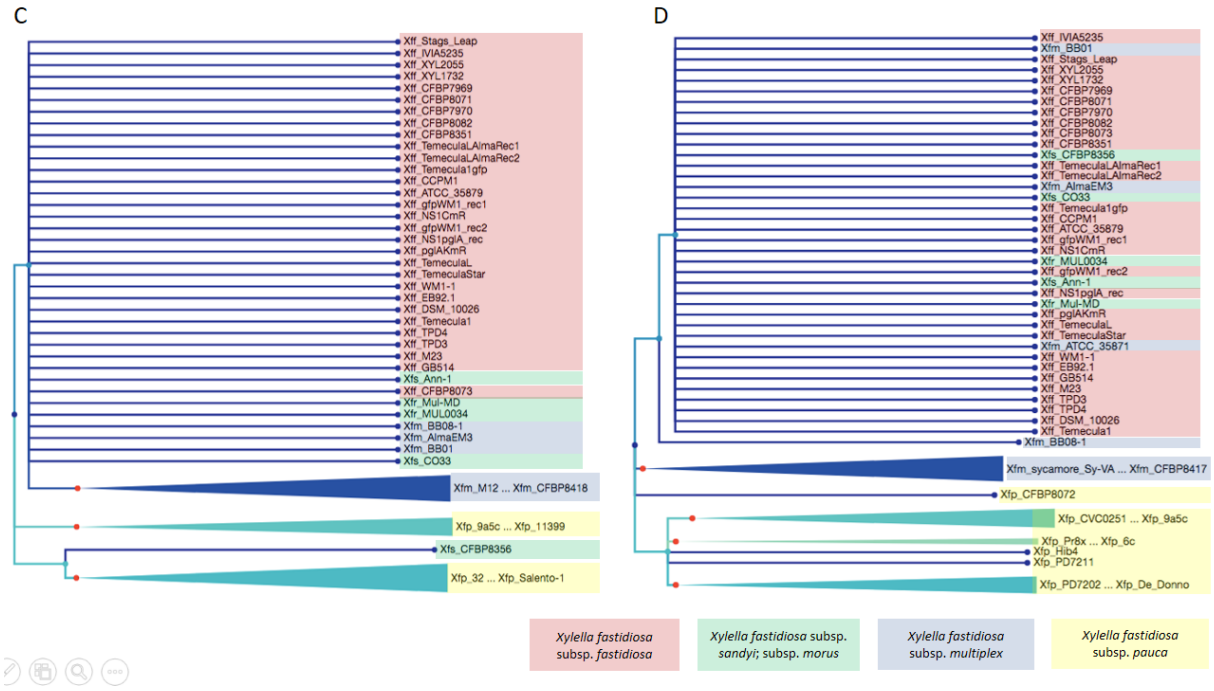


Fig. 1. Phylogenetic trees of Cu-related genes in *X. fastidiosa*.

Phylogenetic trees of *copA* (A); *copB* (B); *copL*(C) and *cutC* genes (D). Maximum-likelihood phylogenetic trees were built using MEGA 7.0, Branches below 70% of bootstraps value were collapsed and collapsed using Phylo.io. For representation purposes, branches were collapsed when the strains belong to same subspecies in a cluster, and genes were color-coded according to the subspecies classification of their strain.

To better understand evolutionary process of the Cu-related genes, Datamonkey.org was used to assess their evolutionary selection. Positive selection increases the diversity and fitness of genes in response to environmental pressure, while negative selection remove these changes (Poon et al. 2009). The results of the analysis showed that when *p*-value threshold was set at 0.1, only *copA* had one codon site under significant positive selection, while other genes did not have any significant positive selected site (Table 1). There were 26 and 19 sites under significant negative

selection in *copA* gene and *copB* gene, respectively. For *copL* gene and *cutC* gene, there were 6 and 4 sites under significant negative selection, respectively, which were less than that of *copA* gene and *copB* gene. If the *p*-value was increased from 0.1 to 0.2, more sites under significant positive selection were detected as well as negative selection. When the *p*-value threshold was 0.2, *copA*, *copB* and *copL* showed positive selected sites while *cutC* still not (Table 1). Based on these results the four Cu-related genes studied here does not seem to be under positive selection during their evolutionary process. However, relevantly strong negative selections were found in these genes. This indicated that there were more random synonymous substitutions in these genes (Delport et al. 2010; Poon et al. 2009). This conservation highlights the importance of these four genes, that they try to keep their function tightly conserved during evolution.

Gene	**# Positive sites (*p*=0.1)**	**# Negative sites (*p*=0.1)**	**# Positives sites (*p*=0.2)**	**# Negative sites (*p*=0.2)**	**# Total sites**
copA	1	26	6	47	611
copB	0	19	2	38	250
copL	0	6	1	14	126
cutC	0	4	0	11	246

Table 1. Summary of selection in the Cu-related genes

Evolutionary selection sites were determined by the analysis carried with Datamonkey.org with Fixed Effects Likelihood method (FEL). Among the results positive selection is considered when non-synonymous substitution (dN) is higher than random synonymous substitution (dS) (dN/dS > 1); negative sites when dN < dS (dN/dS < 1). Total sites is the total codon sites that possibly under selection searched by FEL method.

Cu MIC of *Xylella fastidiosa* strains

From our DNA sequence analysis, we concluded that the four Cu-related genes generally followed the classification of *X. fastidiosa* subspecies. Whether this pattern correlated with Cu resistant ability was studied next. Here, the minimum inhibitory concentration (MIC) of $CuSO_4$ were determined for 55 *X. fastidiosa* strains. From the results (Fig.2), Cu MIC of *X. fastidiosa* subsp. *fastidiosa* ranged between 250 to 450 µM. The Cu MIC of *X. fastidiosa* subsp. *multiplex* ranged between 100 to 350 µM. Due to the quarantine restrictions, only two strains from *X. fastidiosa* subsp. *pauca* were available for our studies. The Cu MIC of *X. fastidiosa* subsp. *pauca* strain De Donno and 1961 were 350 and 200 µM, respectively. The Cu MIC of *X. fastidiosa* subsp. *sandyi* strain MED PRI 047 and Ann-1 were 400 and 800 µM respectively. Cu MIC for Ann-1 was extremely high compared with other strains.

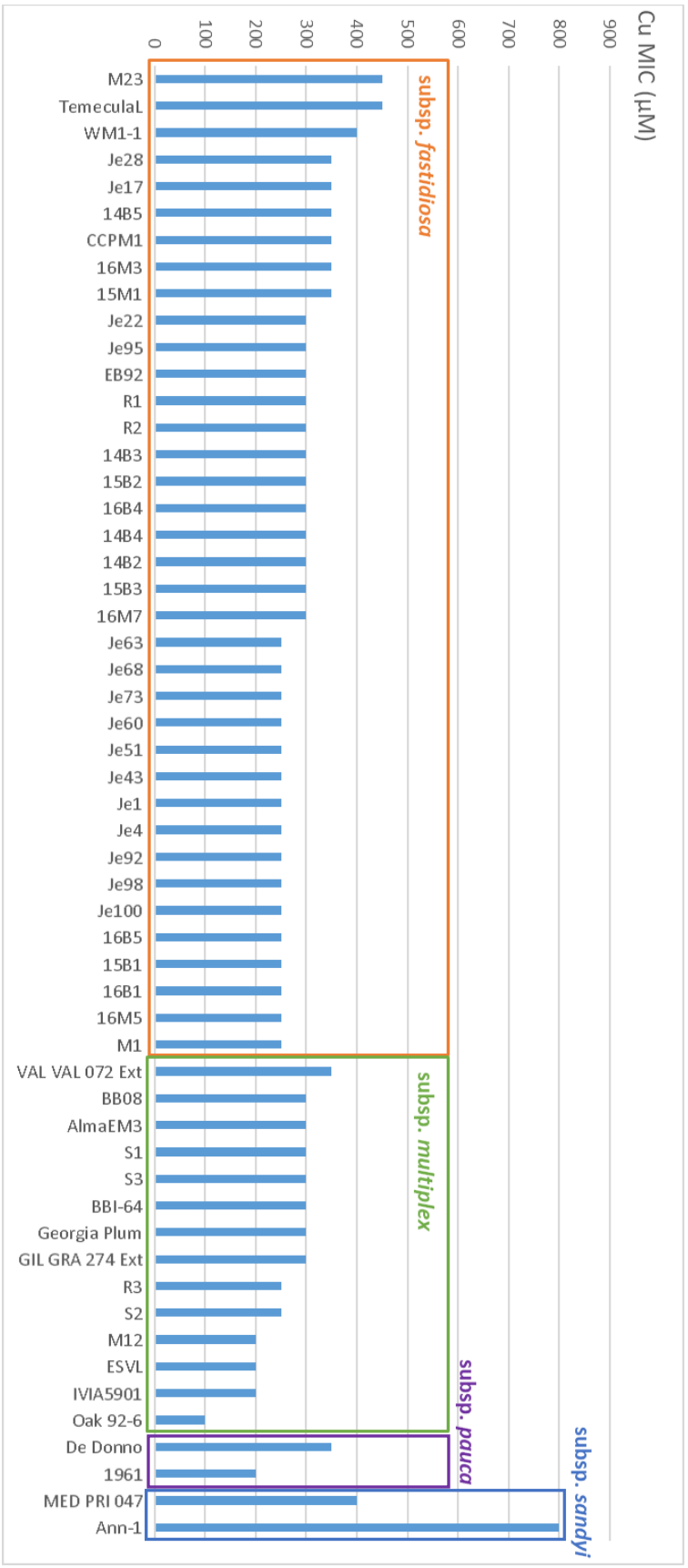

Cu MIC (µM)
0
100
200
300
400
500
600
700
800
900
M23
TemeculaL
WM1-1
Je28
Je17
14B5
CCPM1
16M3
15M1
Je22
Je95
EB92
R1
R2
14B3
15B2
16B4
14B4
14B2
15B3
16M7
Je63
Je68
Je73
Je60
Je51
Je43
Je1
Je4
Je92
Je98
Je100
16B5
15B1
16B1
16M5
M1
VAL VAL 072 Ext
BB08
AlmaEM3
S1
S3
BBI-64
Georgia Plum
GIL GRA 274 Ext
R3
S2
M12
ESVL
IVIA5901
Oak 92-6
De Donno
1961
MED PRI 047
Ann-1
subsp. fastidiosa
subsp. multiplex
subsp. pauca
subsp. sandyi

Fig. 2. Cu MIC of *X. fastidiosa* strains

Cu MIC assessment of *X. fastidiosa* strains were carried in 96-wells plates with PD2 media amended with different concentrations of $CuSO_4$. The initial OD_{600nm} of bacteria culturing was set as 0.01. PD2 media without any bacteria was consider as blank. Growth of bacteria was determined by total growth summed up by biofilm growth and planktonic growth which measured by OD_{600nm}. The minimal Cu concentration that significantly ($p < 0.05$) inhibit bacteria total growth compared with the growth under PD2 media was selected as Cu MIC. Three independent experiments were carried in this study with a total of n=9 replications.

To understand if there was significant differences in Cu MIC among different subspecies groups that were tested, Kruskal-Wallis test was used. From the results (Fig. 3A), the mean rank scores from the test of the Cu MIC data in others (subsp. *pauca* and subsp. *sandyi*), subsp. *fastidiosa* and subsp. *multiplex* were 35, 28 and 24, respectively. Mean rank scores were different in subspecies groups, however, they were not statistically different ($p = 0.4864$) (Fig.3A). Therefore, the Cu MIC of each group of subspecies of *X. fastidiosa* was not significantly differentiated from each other. Furthermore, the relationship between Cu MIC and *X. fastidiosa* strains features, including host and location were studied (strains information is listed in supplemental table 1). Based on the results of Kruskal-Wallis test, similar conclusions could be drawn, different mean rank scores were found among the host and location groups (Fig. 3B, C). However, in both cases *p*-values were bigger than 0.05, therefore no significant differences were found.

Based on the results of the 55 strains tested in this study, no significant correlation was found between their Cu resistant ability and their features including subspecies, host or location.

However, the limitation in this study was that the sample size in each group was uneven, due to the limited availability of *X. fastidiosa* strains from specific groups. It is still possible that some relationships exist between the Cu resistance and isolates characteristics, that could be revealed with a more comprehensive study. In addition, the soil Cu content in a region could be a dominate feature that influence Cu resistance of *X. fastidiosa* isolated from that region. However, lacking such information at the vineyard/farm level could be an obstacle to reveal patterns of Cu resistance among different *X. fastidiosa* strains.

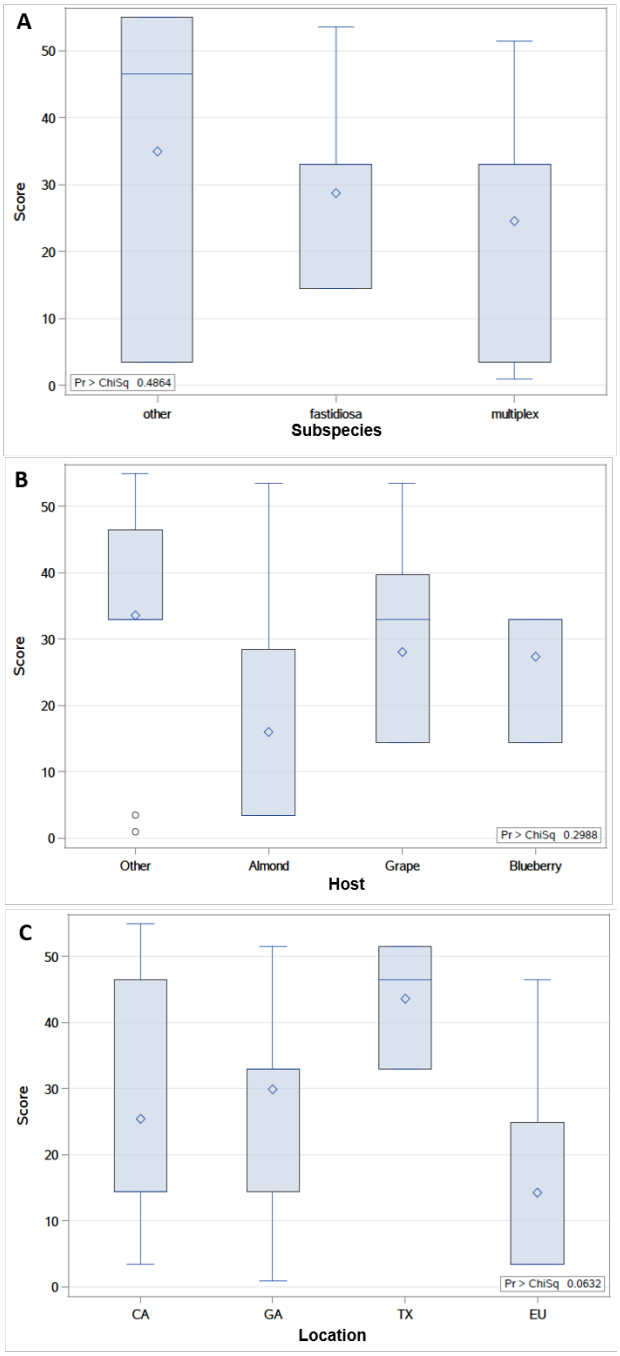


Fig. 3. Rank score of correlation test between Cu MIC and strains features

Rank scores of Kruskal-Wallis test (nonparametric ANOVA) carried by SAS was used to indicate if the Cu MIC from each group were different from each other. Strains were grouped based on their (A) subspecies: fastidiosa (*X. fastidiosa* subsp. *fastidiosa*), multiplex (*X.*

fastidiosa subsp. *multiplex*) and other (*X. fastidiosa* subsp. *pauca* and *X. fastidiosa* subsp. *sandyi)*; (B) host: grape, blueberry, almond and other (olive, oleander, elderberry, plum, giant ragweed, sunflower and oak); (C) location: CA (California), GA (Georgia and Florida), TX (Texas) and EU (Spain and Italy). *p* value (Pr > ChiSq) < 0.05 consider as threshold for statistical significance.

Cu accumulation in *X. fastidiosa* isolates from Georgia's vineyards

Although the above results did not indicate a significant correlation between Cu resistance ability and strains features, a further investigation on a smaller group of *X. fastidiosa* strains was carried. *X. fastidiosa* isolated from grapevines grown in vineyards in the same county in Georgia were selected to be studied. Cu accumulation in cells of the isolates with or without Cu-amended treatments were assessed. *X. fastidiosa* type strain TemeculaL (isolated from grapevines in California) was used as a control. The isolates and control strain were grown in PD2 media, and PD2 media amended with 50μM $CuSO_4$. Cu accumulation in cells of each isolate were measured by ICP-OES. Results (Fig. 4A) indicated that most of the isolates from GA had slightly higher Cu accumulation in cells than TemeculaL, with two isolates (14B7 and 16B4) showing statistically significant differences (p <0.05), compared to control strain when grown in PD2 media. The difference between the isolates and control strain was more pronounced under Cu-amended conditions. Cu accumulation in cells of the isolates were obviously higher than that of control, with four isolates significantly different (p <0.05) (Fig. 4B). Overall, the isolates from Georgia's vineyards accumulated more Cu than control strain both under normal condition (PD2 media) and Cu-amended condition (PD2 amended with 50μM $CuSO_4$). However, there were some exceptions; for example, WM1-1 had lower or similar Cu content in cells as control strain

under the same conditions. WM1-1 (Parker et al., 2012), was isolated form the same region as the other GA isolates used here, but a few years earlier. Maybe the use in the laboratory for several years could be a factor on this difference. Another possible reason could be the difference of antimicrobial and management method used in vineyards, WM1-1 was from Wolf Mountain Vineyard, and the others were from Blackstock Vineyard and Montaluce Vineyard.

When considering the Cu MIC results of the GA isolates (Fig.2), most of the isolates had Cu MIC ranged between 250 to 300 μM. Only 3 isolates Cu MIC were 350 μM and 1 isolate Cu MIC was 400 μM. While, the Cu MIC of control strain was 450 μM. Generally, the isolates from Georgia's vineyards were more sensitive to Cu than control strain TemeculaL. The results of Cu accumulation and Cu MIC are consistent with each other, since more Cu accumulation in cells lead to toxicity build up, and this lower the Cu MIC for the isolates. Moreover, TemeculaL comes from vineyards with longer history in CA, while vineyards in GA are newer. Researchers point out that Cu content in vine and soil are closely related with the aging time of vineyards (Komárek et al. 2010; Ruyters et al. 2013). It is highly possible that GA isolates were less exposed to Cu compared with TemeculaL, which make them more sensitive to Cu as the results showed here. Researchers pointed a clear genomic difference and grouping between whole genomes of CA and GA isolates (A. Castillo, personal communication). However, we did not find the difference between CA and GA isolated in the four Cu-related genes discussed in this study (data not showed). It is interest to know that CA and GA isolates had slightly difference on the phenotype of Cu MIC, but it was not clearly correlated with *copA*, *copB*, *copL* and *cutC* genes phylogeny.

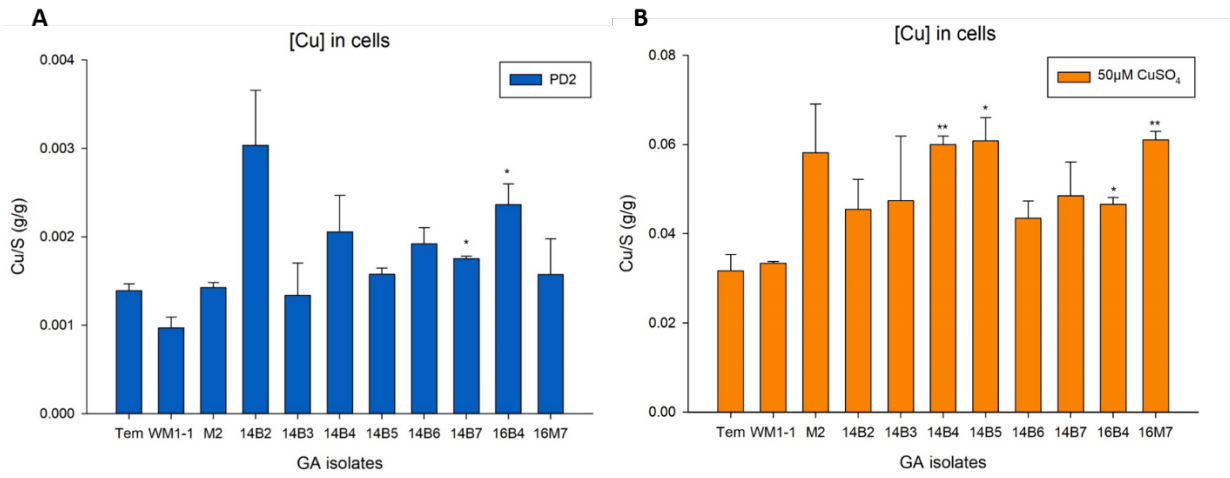


Fig. 4. Cu accumulation in the isolates from Georgia's vineyards

Cu accumulation in cells when grown in PD2 media (A) or PD2 amended with 50μM $CuSO_4$ (B). Cu content was measured by ICP-OES. Isolates were grown in test tubes for 7 days with PD2 media or PD2 amended with 50μM $CuSO_4$. Mean values are shown in graph, and error bars represent standard error of the mean (n=6). Different Y scales were used in this figure since values in A were between 0-0.004; while values in B were between 0-0.08. Data used in the graph corresponds to one representative experiment, and three independent experiments performed under the same conditions showed similar tendencies. * and ** indicate significant differences ($p < 0.05$ and $p < 0.01$, respectively) compared with control strain TemeculaL ('Tem') according to two-tailed Student's *t-test*.

Biofilm formation of *X. fastidiosa* isolated from Georgia's vineyards under Cu treatment

Biofilm formation is one of the key virulence factors of *X. fastidiosa*. We investigated how *X. fastidiosa* isolates from Georgia's vineyards respond to Cu in regards to biofilm formation. Biofilm formation of the isolates under PD2 and PD2 amended with 50 μM $CuSO_4$ conditions were analyzed and compared to control strain TemeculaL. It was shown in the results that most

of the isolates had higher biofilm/total cells ratios when grown in PD2 media, except isolates M2, 14B2 and 14B3, which had lower biofilm/total cells ratio. When grown in Cu-amended media, increase of biofilm/total cells ratios were noticed in all the isolates, with 4 isolates showing significant increase (p <0.05). 14B3 showed significantly lower biofilm/total cells ratio compared with other isolates and control strain, even under Cu-amended condition. While, 14B3 was not significantly different from other strains in Cu MIC assessment, nor Cu accumulation in cells. The causing reason of its low biofilm/total cells ratio in all the tested conditions may not be related with Cu homeostasis, as it has the same response to Cu amendments as other isolates. Our results showed that Cu could not only induced biofilm cell growth as it is showed in previous research (Cobine et al. 2013), but more importantly, increased the proportions that biofilm cells in comparison to total cells, which potentially increase *X. fastidiosa* in planta fitness and virulence. Meanwhile, the overall increase of biofilm/total cells ratio may related with the metabolism consequences of increased Cu accumulation in cells under Cu-amended condition.

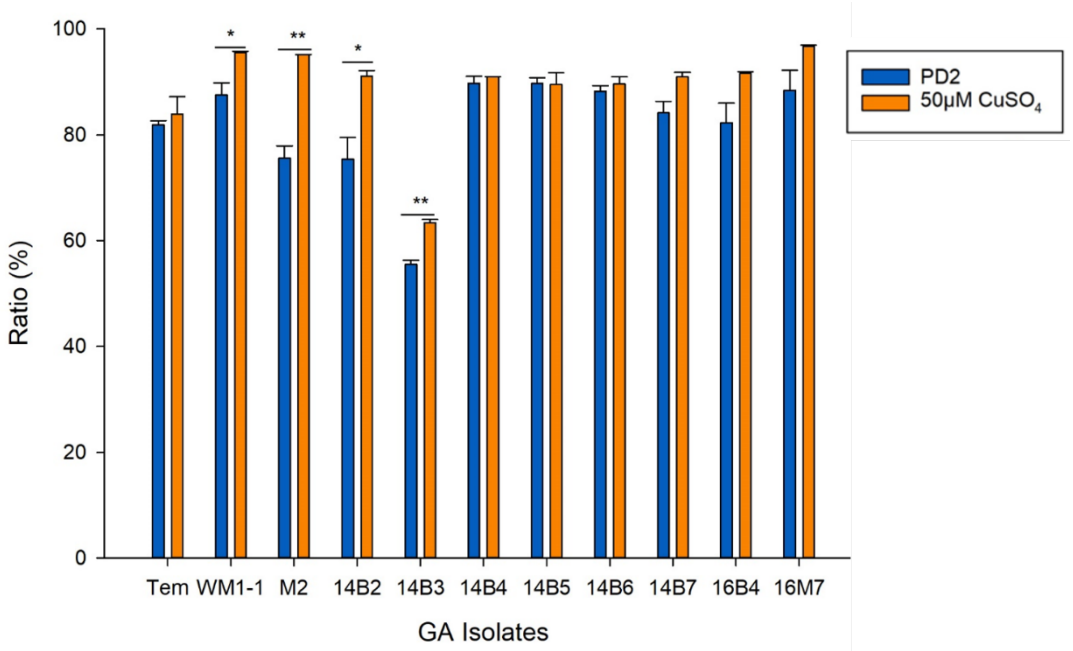


Fig. 5. Biofilm/total cells ratios of the isolates from Georgia's vineyards

Biofilm/total cells ratio was measured by the sulfur (S) concentration in biofilm cells divided by S concentration in total cells, as cellular S content is linearly correlated with cell number. Bacteria were grown under PD2 media or PD2 amended with 50μM $CuSO_4$. After 7 days of culturing under PD2 media or PD2 media amended with 50 μM $CuSO_4$, biofilm cells and planktonic cells were carefully separated with pipetting and collected by centrifugation. Data used in the graph corresponds to one representative experiment, and three independent experiments performed under the same conditions showed similar tendencies. * and ** above the black line indicate significant differences ($p < 0.05$ and $p < 0.01$, respectively) of biofilm/ total cells ration when grown in PD2 media and PD2 media amended with 50 μM $CuSO_4$ according to two-tailed Student's *t-test*.

Conclusion

In this chapter, we investigated which features would possibly influence Cu resistant ability of *X. fastidiosa*. The phylogeny analysis of *copA*, *copB*, *copL* and *cutC* indicated that these Cu-related genes roughly followed *X. fastidiosa* subspecies classification. *copL* was more conserved than other three genes, and since this is a regulatory gene, it may indicate that the Cu resistance/homeostasis in this bacterium is well conserved. None of the genes studied here was under positive selection during their evolutionary process, further supporting this observation. To find if Cu resistant ability of *X. fastidiosa* strains were correlated with this phylogeny, Cu MIC of *X. fastidiosa* strains were assessed and compared with its phylogeny. However, no statistically significant correlation with phylogeny was found. Further studies also did not find correlations of Cu resistant ability with either host or location. We then focus our investigation on Cu influence

on a group of *X. fastidiosa* that all were isolated from vineyards in Georgia. These isolates, obtained from the same host (grapevine) and same location (Dahlonega, Georgia), mostly responded similarly to Cu amendments. However, these responses were not correlated for each isolate. In general, these isolates had higher Cu accumulation in cells and lower Cu MIC than control strain (TemeculaL). A buildup of toxicity from Cu accumulation would lead to hypersensitive to Cu in these isolates. Based on our preliminary data, the variability in Cu resistant ability is not explained by either location, host or phylogeny of *X. fastidiosa* strains, since no correlation was found between them. Limited and uneven sample size on each feature group, due to limited availability of *X. fastidiosa* strains, might have been a problem to answer our questions. Further research with more strains could help us better understand the importance of Cu resistant ability to the *X. fastidiosa* strains from different geographic locations or host species. By study this, we are hoping to understand the adaptation of *X. fastidiosa* to different environments, and to help providing novel ideas for worldwide *X. fastidiosa* control and its disease management.

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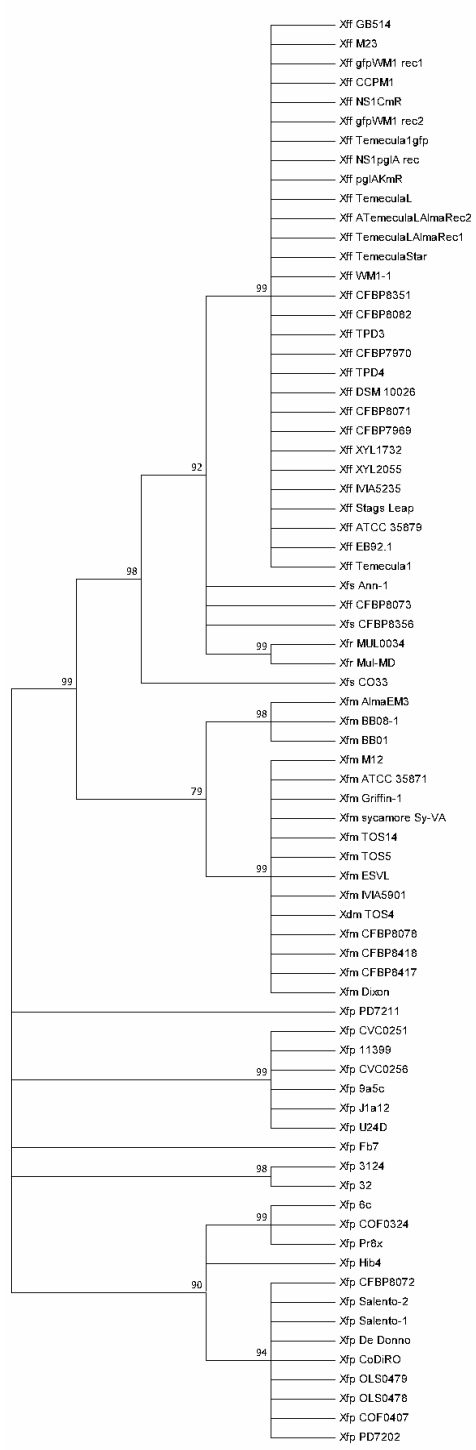
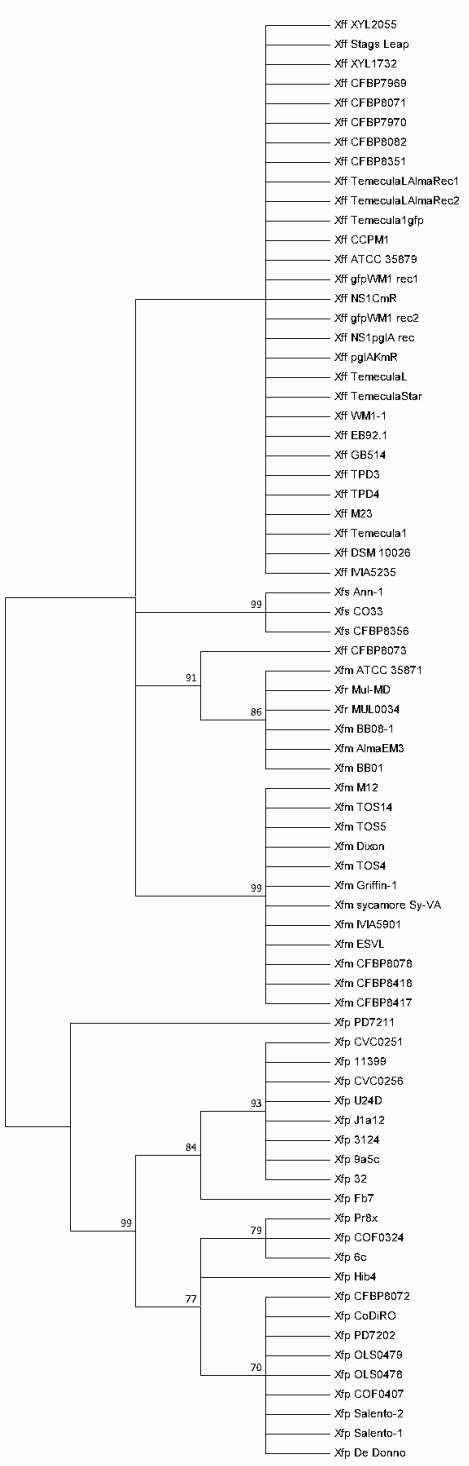
Supplemental Information

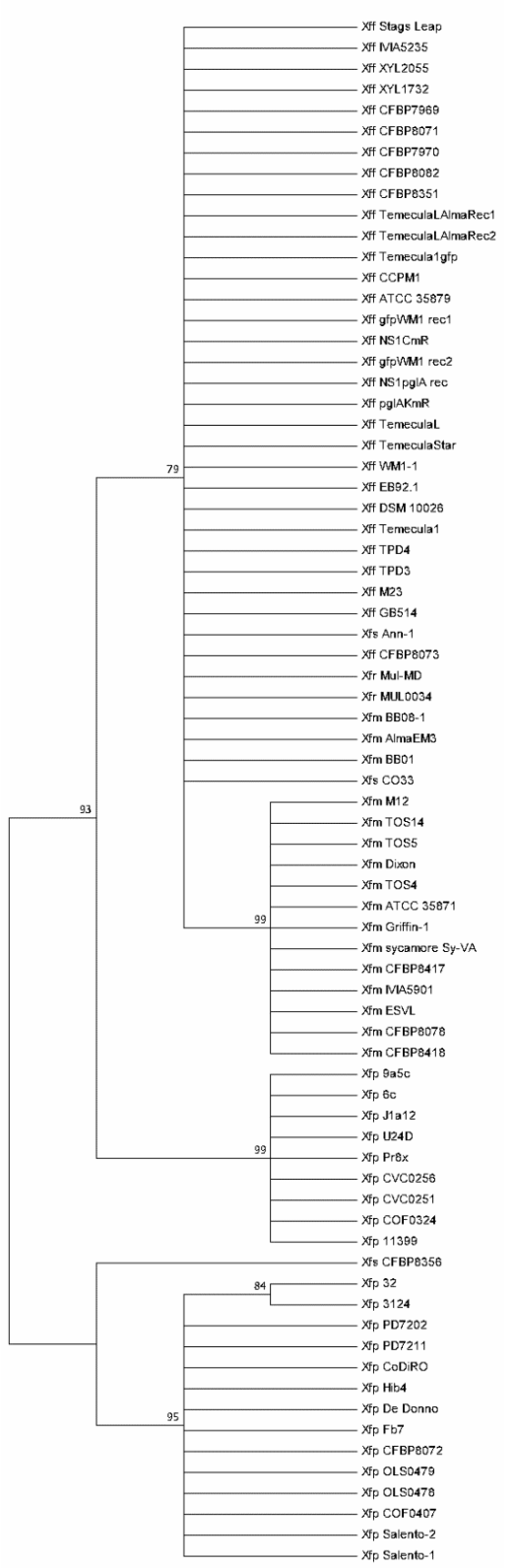
Supplemental figure 1: Phylogenetic trees of *copA*, *copB*, *copL* and *cutC*

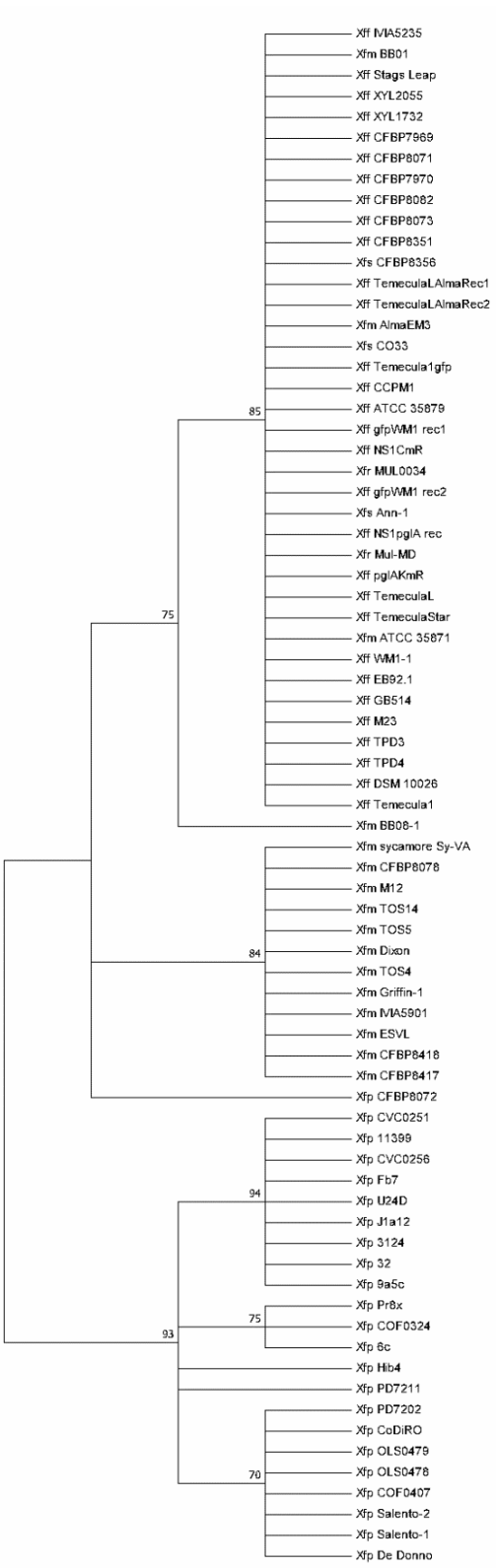


copA phylogenetic tree



copB phylogenetic tree





copL phylogenetic tree *cutC* phylogenetic tree

Phylogenetic trees of *copA*, *copB*, *copL* and *cutC* without collapsing strain names classified in the same cluster.

Phylogenetic trees of *copA*; *copB*; *copL* and *cutC* genes. Maximum-likelihood phylogenetic trees were built using MEGA 7.0, Branches below 70% of bootstraps value were collapsed. For representation purposes, branches were collapsed when the strains belong to same subspecies in a cluster. The strains were named according to subspecies classification as follows: Xff: *X. fastidiosa* subsp. *fastidiosa*; Xfm: *X. fastidiosa* subsp. *multiplex*; Xfs: *X. fastidiosa* subsp. *sandyi*; Xfr: *X. fastidiosa* subsp. *morus*; Xfp: *X. fastidiosa* subsp. *pauca*.

Supplemental table 1: Information of *X. fastidiosa* isolates and strains for Cu MIC assessment.

Stains	**Cu MIC (µM)**	**Subspecies**	**State**	**Location**	**Host**	**Host detail**	**Isolate Sources**
M23	450	*fastidiosa*	CA	Kern	Almond	Rosaceae, Prunus dulcis	6
TemeculaL	450	*fastidiosa*	CA	Temecula, Riverside	Grape	Vitaceae, Vitis vinifera	1
WM1-1	400	*fastidiosa*	GA	Wolf Mountain, Dahlonega	Grape	Mourvedre	1
Je28	350	*fastidiosa*	CA	Napa, Spring Mountain	Grape	Merlot	2
Je17	350	*fastidiosa*	CA	Sonoma, Newsome	Grape	Malbec	2
14B5	350	*fastidiosa*	GA	Blackstock, Dahlonega	Grape	Viognier	1
CCPM1	350	*fastidiosa*	GA	Cavender Creek, Dahlonega	Grape	Petite Manseng	1
16M3	350	*fastidiosa*	GA	Montaluce, Dahlonega	Grape	Merlot	1
15M1	350	*fastidiosa*	GA	Montaluce, Dahlonega	Grape	Montaluce	1
Je22	300	*fastidiosa*	CA	Napa, Spring Mountain	Grape	Merlot	2
Je95	300	*fastidiosa*	CA	Sonoma, Rudd	Grape	Chardonnay	2
EB92	300	*fastidiosa*	FL	Leesburg, Lake	Elderberry	Sambucus canadensis	4
R1	300	*fastidiosa*	GA	Bacon, Blake Williams Farm	Blueberry	Rebel	1
R2	300	*fastidiosa*	GA	Bacon, Blake Williams Farm	Blueberry	Rebel	1
14B3	300	*fastidiosa*	GA	Blackstock, Dahlonega	Grape	Cab Sauvignon	1

15B2	300	*fastidiosa*	GA	Blackstock, Dahlonega	Grape	Chardonnay	1
16B4	300	*fastidiosa*	GA	Blackstock, Dahlonega	Grape	Chardonnay	1
14B4	300	*fastidiosa*	GA	Blackstock, Dahlonega	Grape	Chardonnay	1
14B2	300	*fastidiosa*	GA	Blackstock, Dahlonega	Grape	Mourvedre	1
15B3	300	*fastidiosa*	GA	Blackstock, Dahlonega	Grape	Viognier	1
16M7	300	*fastidiosa*	GA	Montaluce, Dahlonega	Grape	Vidal	1
Je63	250	*fastidiosa*	CA	Bakersfield, Flames	Grape	Flames	2
Je68	250	*fastidiosa*	CA	Bakersfield, Flames	Grape	Flames	2
Je73	250	*fastidiosa*	CA	Bakersfield, Red Globe	Grape	Red Globe	2
Je60	250	*fastidiosa*	CA	Bakersfield, Scarlet Royal	Grape	Scarlet Royal	2
Je51	250	*fastidiosa*	CA	Napa, Silverado	Grape	Merlot	2
Je43	250	*fastidiosa*	CA	Napa, Yount Mill	Grape	Cabernet Sauvignon	2
Je1	250	*fastidiosa*	CA	Santa Barbara	Grape	Chardonnay	2
Je4	250	*fastidiosa*	CA	Santa Barbara	Grape	Pinot Noir	2
Je92	250	*fastidiosa*	CA	Sonoma, MacMurray	Grape	Gruner Veltliner	2
Je98	250	*fastidiosa*	CA	Temecula	Grape	N/A	2
Je100	250	*fastidiosa*	CA	Temecula	Grape	N/A	2
16B5	250	*fastidiosa*	GA	Blackstock, Dahlonega	Grape	Chardonnay	1
15B1	250	*fastidiosa*	GA	Blackstock, Dahlonega	Grape	Merlot	1
16B1	250	*fastidiosa*	GA	Blackstock, Dahlonega	Grape	Merlot	1
16M5	250	*fastidiosa*	GA	Montaluce, Dahlonega	Grape	Pinot Noir	1
M1	250	*fastidiosa*	GA	Pierce, Stevie Yong Farm	Blueberry	Meadowlark	1
VAL VAL 072 Ext	350	*multiplex*	TX	Del Rio, Val Verde	Giant Ragweed	Ambrosia trifida var. texana	3

BB08	300	*multiplex*	FL	Palatka, Putnam	Blueberry	Star	4
AlmaEM3	300	*multiplex*	GA	Alma	Blueberry	Emerald	1
S1	300	*multiplex*	GA	Bacon, Blake Williams Farm	Blueberry	Star	1
S3	300	*multiplex*	GA	Bacon, Blake Williams Farm	Blueberry	Star	1
BBI-64	300	*multiplex*	GA	N/A	Blueberry	N/A	5
Georgia Plum	300	*multiplex*	GA	N/A	Plum	Rosaceae, Prunus sp.	2
GIL GRA 274 Ext	300	*multiplex*	TX	Willow City, Gillespie	Annual Sunflower	Asteraceae, Helianthus annuus	3
R3	250	*multiplex*	GA	Bacon, Blake Williams Farm	Blueberry	Rebel	1
S2	250	*multiplex*	GA	Bacon, Blake Williams Farm	Blueberry	Star	1
M12	200	*multiplex*	CA	Kern	Almond	Rosaceae, Prunus dulcis	6
ESVL	200	*multiplex*	Spain	Alicante	Almond	N/A	8
IVIA5901	200	*multiplex*	Spain	Alicante	Almond	N/A	8
Oak 92-6	100	*multiplex*	FL	Palm Beach	Oak	Quercus sp.	4
De Donno	350	*pauca*	Italy	Apulia	Olive	Olea europaea L.	9
1961	200	*pauca*	Spain	Ibiza	Olive	Wild olives	8
Ann-1	800	*sandyi*	CA	N/A	Oleander	N/A	2
MED PRI 047	400	*sandyi*	TX	Median	Oleander	N/A	3

N/A = information non-available

Isolate Sources: 1: Leonardo De La Fuente (Auburn University), 2: Rodrigo Almeida (University of California, Berkeley), 3: Mark Black (Texas A&M University), 4: Donald Hopkins (University of Florida), 5: Harald Scherm (University of Georgia), 6: Jianchi Chen (USDA, Parlier, CA), 7: C.J. Chang (University of Georgia), 8, Blanca Landa (CSIC-IAS, Spain), 9. Maria Saponari (CNR, Italy)

Supplemental table 2: Information of *X. fastidiosa* isolates and strains for phylogeny

Strain	Host plant	Place of isolation	Reference	GenBank Accession
Xylella fastidiosa* subsp. *fastidiosa				
ATCC 35879	Grape	USA (FL)	(Chen et al. 2020)	JQAP00000000.1
CCPM1	Grape	USA (GA)	(Parker et al. 2012)	PUJB00000000.1
CFBP7969	Grape	USA(NC)	(Denancé et al. 2019)	PHFQ00000000.1
CFBP7970	Grape	USA (FL)	(Denancé et al. 2019)	PHFR00000000.1
CFBP8071	Almond	USA (CA)	(Denancé et al. 2019)	PHFP00000000.1
CFBP8073	Coffee	France	(Jacques et al. 2016)	LKES00000000.1
CFBP8082	Annual ragweed	USA (FL)	(Denancé et al. 2019)	PHFT00000000.1
CFBP8351	Grape	USA (CA)	(Denancé et al. 2019)	PHFU00000000.1
DSM 10026	Grape	USA (FL)	(Varghese JN, unpublished)	FQWN00000000.1
EB92.1	Elderberry	USA (FL)	(Zhang et al. 2011)	AFDJ00000000.1
GB514	Grape	USA (TX)	(Schreiber et al. 2010)	CP002165.1
gfpWM1-1 Rec1	Recombinants of WM1-1 with KLN59.3 donor	In vitro	(Kandel et al. 2017)	PUJD00000000.1
gfpWM1-1 Rec2	Recombinants of WM1-1 with KLN59.3 donor	In vitro	(Kandel et al. 2017)	PUJE00000000.1
IVIA5235	Cherry	Spain	(Landa et al. 2018)	CP047171.1
M23	Almond	USA (CA)	(Chen et al. 2010)	CP001011.1
NS1-CmR	Mutant of TemeculaL	In vitro	(Matsumoto et al. 2009)	PUJF00000000.1
NS1pglA Rec	Recombinant of NS1-CmR and pglA-KmR	In vitro	(Kandel et al. 2016)	PUJG00000000.1
pglA-KmR	Grape	USA (CA)	(Roper et al. 2007)	PUJH00000000.1
Stag's Leap	Grape	USA (CA)	(Chen et al. 2016)	LSMJ00000000.1
Temecula1	Grape	USA (CA)	(Van Sluys et al. 2003)	PUJI00000000
Temecula1gfp	Mutant of Temecula1	In vitro	(Newman et al. 2003)	PUJC00000000.1
TemeculaL	Grape	USA (CA)	(Potnis et al. 2019)	PUJJ00000000.1
TemeculaLAlmaRec1	Recombinant of TemeculaL with AlmaEM3 donor	In vitro	(Potnis et al. 2019)	PUIW00000000.1
TemeculaLAl	Recombinant of TemeculaL	In vitro	(Potnis et al. 2019)	PUIX00000

maRec2	with AlmaEM3 donor			000.1
TemeculaStar	Grape	USA (GA)	(Potnis et al. 2019)	PUJI00000000.1
TPD3	Grape	Taiwan	(Castillo et al. 2019)	VJWG00000000.1
TPD4	Grape	Taiwan	(Castillo et al. 2019)	VJWH00000000.1
WM1-1	Grape	USA (GA)	(Parker et al. 2012)	PUJK00000000.1
XYL1732	Grape	Spain	(Gomila et al. 2019)	QTJT00000000.1
XYL2055	Grape	Spain	(Gomila et al. 2019)	QTJS00000000.1
Xylella fastidiosa* subsp. *morus				
MUL0034	Mulberry	USA (CA)	(Schuenzel et al. 2005)	CP006740.1
Mul-MD	Mulberry	USA (MD)	(Guan et al. 2014)	AXDP00000000.1
Xylella fastidiosa* subsp. *sandyi				
CFBP8356	Coffee	Costa Rica	(Denancé et al. 2019)	PHFV00000000.1
Ann-1	Oleander	USA (CA)	(Schuenzel et al. 2005)	AAAM00000000.4
CO33	Coffee	Italy	(Giampetruzzi et al. 2015a)	LJZW00000000.1
Xylella fastidiosa* subsp. *multiplex				
AlmaEM3	Blueberry (Emerald)	USA (GA)	(Oliver et al. 2014)	PUIY00000000.1
BB01	Blueberry	USA (GA)	(Van Horn et al. 2017)	MPAZ00000000.1
BB08-1	Blueberry	USA (FL)	(Oliver et al. 2014)	PUIZ00000000
CFBP8078	Periwinkle	USA (FL)	(Denancé et al. 2019)	PHFS00000000.1
CFBP8417	Spanish Broom	France	(Denancé et al. 2019)	LUYB00000000.1
CFBP8418	Spanish Broom	France	(Denancé et al. 2019)	LUYA00000000.1
Dixon	Almond	USA (CA)	(Bhattacharyya et al. 2002)	AAAL00000000.2
ESVL	Almond	Spain	(Giampetruzzi et al. 2019b)	QPQV00000000.1
IVIA5901	Almond	Spain	(Landa et al. 2018)	CP047134.1
M12	Almond	USA (CA)	(Chen et al. 2010)	CP000941
sycamore Sy-VA	Sycamore	USA (VA)	(Guan et al. 2014)	JMHP00000000.1
TOS14	Spanish Broom	Italy	(Giampetruzzi et al.	SMTJ00000

			2019a)	000.1
TOS4	Almond	Italy	(Giampetruzzi et al. 2019a)	SMTH00000000.1
TOS5	Myrtle-leaf milkwort	Italy	(Giampetruzzi et al. 2019a)	SMTI00000000.1
ATCC 35871	Hybrid Plum	USA (GA)	(Kyrpides et al, unpublished)	AUAJ00000000.1
Griffin-1	Oak	USA (GA)	(Chen et al. 2013)	AVGA00000000.1
Xylella fastidiosa* subsp. *pauca				
3124	Coffee	Brazil (São Paulo)	(Li et al. 2001)	CP009829.1
CFBP8072	Coffee	France	(Jacques et al. 2016)	LKDK00000000.1
CoDiRO	Olive	Italy (Apulia)	(Giampetruzzi et al. 2015b)	JUJW00000000.1
De Donno	Olive	Italy (Apulia)	(Giampetruzzi et al. 2017)	CP020870.1
PD7202	Coffee	Netherlands	(Bergsma-Vlami et al. 2017)	RRUA00000000.1
PD7211	Coffee	Netherlands	(Bergsma-Vlami et al. 2017)	RRTZ00000000.1
32	Coffee	Brazil (São Paulo)	(Grisard et al. 2014)	AWYH00000000.1
11399	Orange	Brazil	(Niza et al. 2016)	JNBT00000000.1
6c	Coffee	Brazil (São Paulo)	(Grisard et al. 2014)	AXBS00000000.2
9a5c	Sweet orange	Brazil (São Paulo)	(Simpson et al. 2000)	AE003849.1
COF0324	Coffee	Brazil	(Knight et al, 2017, unpublished)	LRVG00000000.1
COF0407	Coffee	Costa Rica	(Knight et al, 2017, unpublished)	LRVJ00000000.1
CVC0251	Sweet orange	Brazil	(Knight et al, 2017, unpublished)	LRVE00000000.1
CVC0256	Sweet orange	Brazil	(Knight et al, 2017, unpublished)	LRVF00000000.1
Fb7	Sweet orange	Argentina (Corrientes)	(da Silva et al. 2007)	CP010051.2
Hib4	Hibiscus	Brazil (São Paulo)	(Pierry and da Silva, 2017, unpublished)	CP009885.1
J1a12	Sweet orange	Brazil (São Paulo)	(Pierry and da Silva, 2017, unpublished)	CP009823.1
OLS0478	Oleander	Costa Rica	(Knight et al, 2017, unpublished)	LRVI00000000.1
OLS0479	Oleander	Costa Rica	(Knight et al, 2017, unpublished)	LRVH00000000.1
Pr8x	Plum	Brazil (São	(Pierry and da Silva	CP009826.1

		Paulo)	2015, unpublished)	
Salento-1	Olive	Italy (Apulia)	(Bleve et al. 2016)	CP016608.1
Salento-2	Olive	Italy (Apulia)	(Ramazzotti et al. 2018)	CP016610.1
U24D	Sweet orange	Brazil (São Paulo)	(Da Silva, 2017 unpublished)	CP009790.1

Chapter 6: General conclusions

Xylella fastidiosa, a plant bacterial pathogen, draws broad attention as it can cause many diseases worldwide with significant economic losses. Finding possible control methods against this pathogen is very urgent.

In my dissertation, the possible effect of Cu in controlling *X. fastidiosa* was discussed. It was found that *X. fastidiosa* populations were not inhibited in plants, and disease symptoms were not controlled when additional Cu was amended to plants. Instead, a trend of increasing the disease symptom and bacterial populations were noticed at the end. This was believed to be related with a growth-promoting Cu concentration in the plant xylem system, as the results of plant Cu homeostasis machinery adjustment. Although Cu is a widely used antimicrobial element in the field and often found to reach a high concentration in *X. fastidiosa* living environment, it does not contribute to this pathogen control or disease management, instead, serves as a booster for endophyte pathogens growth. Plant disease management in field should be under carefully monitoring as control methods for one single pathogen may influence non-target microflora.

Further, the study of Cu-related genes in *X. fastidiosa* showed *copA*, *copB* and *cutC* genes are crucial to the full function of Cu resistance/homeostasis in the pathogen and could influence its virulence in plant especially under Cu supplemented condition. The different phenotypes showed in *copA*, *copB* and *cutC* genes knockout mutants reveal that these genes are not functioning in the same way towards Cu resistance/homeostasis (Fig.1). With the help of these Cu-related genes, *X. fastidiosa* could withstand Cu stresses, both chronic and acute, from its living

environment, and this is believed to be related with the evolutionary adaptation to the xylem vessels of plant.

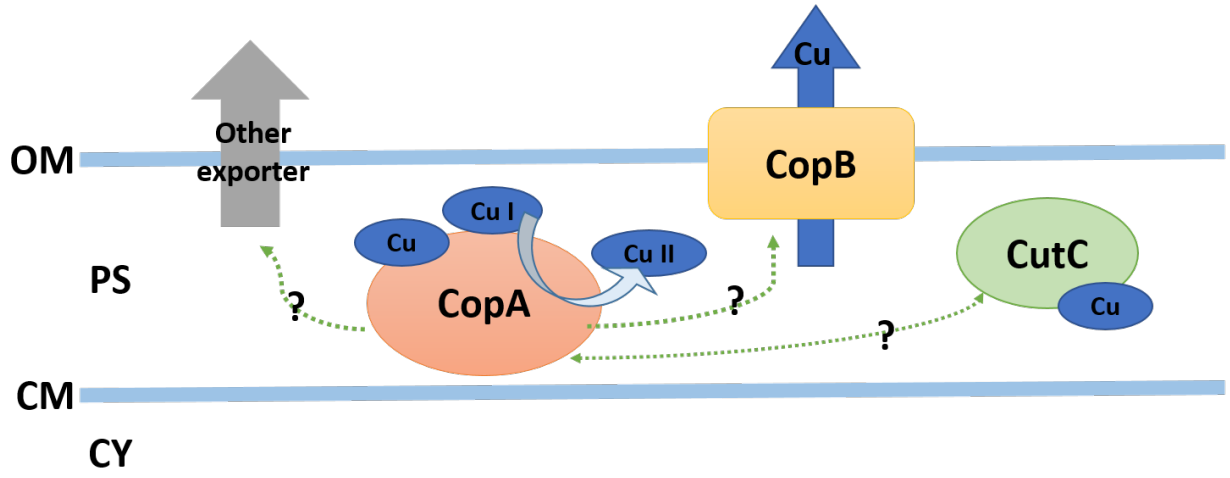


Fig. 1. Hypothetical model of Cu resistance/homeostasis in *X. fastidiosa*

Hypothetical model of Cu resistance/homeostasis in *X. fastidiosa* based on experimental data in this study and references in other plant pathogenic bacteria (Puig et al. 2002; Bondarczuk and Piotrowska-Seget 2013). Hypothetically, in *X. fastidiosa* CopA function is to detoxify extra Cu by oxidizing Cu, while CopB functions as a Cu exporter. CutC function is to detoxify Cu by binding it. Abbreviations: OM: outer membrane, PS: periplasmic space, CM: cytoplasmic membrane, CY: cytoplasm. Arrows dotted lines indicate presumed interactions between proteins

In this dissertation, a correlation between Cu related genes phylogeny, Cu resistance/homeostasis ability and the geography and host features of isolation was not found. It should not be ignored that Cu resistance/homeostasis in *X. fastidiosa* is possibly under the influences of many factors. A possible acquisition of Cu resistance from other bacteria in the environment and spread within the population could be possible, considering the natural competence of *X. fastidiosa*. Searching the factors that determine Cu resistance distribution in *X. fastidiosa* is always necessary and urgent.

Based on the in vitro and in planta studies presented in this dissertation, we have a basic understanding of how Cu influences the virulence of *X. fastidiosa*, and more importantly, the molecular basis of Cu resistance/homeostasis in *X. fastidiosa*. We hope to apply this information to come up with strategies of plant pathogen control and diseases management. Importantly, I advanced the knowledge of Cu resistance/homeostasis in phytopathogens and gram-negative bacteria.