RISK ASSESSMENT OF COPPER AND STREPTOMYCIN RESISTANCE DEVELOPMENT IN *Xanthomonas citri* subsp. *citri*

By

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A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA

2010

To my lovely wife Lidiane and our families for making this possible

ACKNOWLEDGMENTS

I am very thankful to my committee chair Dr. James H. Graham for entrusting me with this work, for sharing his expertise and for the financial support. I would like to especially acknowledge my cochair Jeffrey B. Jones for wisely guiding every step taken throughout the entire research project. My thanks also go to the members of my committee Robert E. Stall, Max Teplitski and James Preston for all their support, constructive criticism and helpful discussions. Special thanks go to Dr. Xiaoan Sun and Debra Jones from the Department of Plant Industry (DPI) in Gainesville, FL; Dr. Rui Leite from the Agronomic Institute of Paraná State (IAPAR), Londrina, Paraná, Brazil; and Dr. Blanca Canteros from Instituto Nacional de Tecnología Agropecuaria, Bella Vista, Corrientes, Argentina, for kindly providing bacterial strains to this study.

During this work I have collaborated with many colleagues from the bacteriology lab for whom I have great regard and I wish to extend my warmest thanks. I would like to thank Aaron Hert, Botond Balogh, Frank Figueiredo, Jason Hong, Mine Hantal and Neha Potnis. I also would like to express my sincere gratitude to Daniele Liberti, Donna Perry, Ellen Dickstein, Gail Harris, Janice Sap, Jerry Minsavage, Lauretta Rahmes, Monty Myers, and Sherri Mizzel, who somehow willingly contributed for the accomplishment of this work.

I lovely and warmly thank my wife Lidiane for being by my side all the time with all the love and belief in me that I could ask, for participating decisively in the accomplishment of this work. Thanks go to our family for blessing our decision to fly higher and constant encouragement. They have been the reference I need to sail in the right direction. Thank you all.

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December 2010

Chair: James H. Graham
Cochair: Jeffrey B. Jones
Major: Plant Pathology

Despite more than two decades (1984-2006) of eradication attempts, citrus canker, caused by *Xanthomonas citri* subsp. *citri* (Xcc), has spread across much of the Florida citrus industry. After eradication efforts were halted in 2006, canker management shifted to disease suppression strategies, including use of topical sprays of copper and streptomycin. One problem with these bactericides is that their widespread use may lead to development of resistance in Xcc. The major objectives of this dissertation were to assess the risk for the development of copper resistant (Cu^R) and streptomycin resistant (Sm^R) Xcc and to characterize and compare the genetics of copper resistance in Xcc with other bacteria. A number of factors favorable for the development of copper resistance in Xcc were identified, but further investigation is necessary to fully assess the risk for streptomycin resistance. Although no Cu^R strains of Xcc were detected in Florida and Brazil, many strains of *Xanthomonas alfalfae* subsp. *citrumelonis* (Xac), the casual agent of citrus bacterial spot in Florida, were resistant to copper. This is the first time copper resistance has been reported in Xac and since Xac and Xcc share the same host and thrive under similar environmental conditions, the

concern is that copper resistance may be horizontally transferred from Xac to Xcc. This concern is supported by experiments that showed that copper resistance genes can be conjugated among different species of *Xanthomonas* including Xcc and Xac. Moreover, although no Cu^R or Sm^R strains of Xcc were isolated from citrus trees repeatedly sprayed with copper or streptomycin for 3 consecutive seasons, the frequent sprays caused an increase in the population of endemic bacteria with resistance to these chemicals. The intensive use of these bactericides may consequently increase the risks for acquisition by Xcc of copper or streptomycin resistance genes from epiphytic bacteria. This possibility is supported by the presence of Xcc copper resistance gene homologues in bacteria from the citrus tree canopy which are able to confer resistance to copper sensitive strains of *Xanthomonas*. Cloning and characterization of copper resistance genes in Xcc revealed *copL, copA* and *copB* as the major determinants of resistance. Homologues of these genes with identity higher than 90% occurred in Cu^R strains of several other species of *Xanthomonas* and other bacterial species, indicating that these copper resistance determinants are widespread and may be transferable into Xcc populations under repeated use of copper for citrus canker management.

CHAPTER 1
COPPER AND STREPTOMYCIN RESISTANCE IN PLANT PATHOGENIC BACTERIA

Introduction

Citrus canker, caused by *Xanthomonas citri* subsp. *citri* (Xcc) (syn. *Xanthomonas axonopodis* pv. *citri*), is a globally distributed, highly contagious bacterial disease of citrus species such as sweet orange, lime, lemon, or grapefruit (Civerolo, 1984; Gottwald et al., 2002; Leite and Mohan, 1990). Moreover, it is one of the most serious diseases of citrus with substantial impact on the citrus production industry, particularly fresh fruit (Leite and Mohan, 1990; Stall and Seymour, 1983). Severe infection results in defoliation, die-back, deformation of fruit and premature fruit drop with infected fruits being less valuable or entirely unmarketable (Civerolo, 1984; Stall and Seymour, 1983). The severity of the infection varies with different species and varieties and the prevailing climatic conditions. Disease symptoms are characterized by raised circular necrotic lesions that develop on leaves, twigs and fruits. On leaves, lesions first have an oily appearance, usually on the abaxial surface, and become evident on both the abaxial and adaxial surfaces of the leaf with age. Older lesions on leaves and fruit tend to have more raised margins with a sunken center and may be surrounded by a yellow chlorotic halo. Sunken centers are especially noticeable on fruits, but the lesions do not penetrate far into the rind thus not affecting internal quality (Civerolo, 1984; Gottwald et al., 2002; Stall and Seymour, 1983).

Control methods for citrus canker in Florida, some of which have been very controversial, have included eradication of trees, quarantine on transportation of fresh fruit and trees within Florida and to citrus producing states, production of Xcc-free nursery trees, and sanitation and decontamination measures (Gottwald et al., 2002).

Despite eradication attempts, by late 2005 the disease had spread across much of the state. In 2004 and 2005 the State and Federal agricultural surveys indicated that the bacterium had probably been spread widely by hurricanes. Thus, in 2006 eradication was suspended and a new policy was established (Gottwald and Irey, 2007; Irey et al., 2006). Hence, in Florida, the current efforts are focused on developing new strategies for managing citrus canker.

In the short term, foremost of the strategies for control of citrus canker is the optimization of copper sprays, such as number, timing and frequency of sprays (Behlau et al., 2010), product rate and formulation. Additionally, alternative materials, such as streptomycin, have been tested as a complementary measure to augment copper bactericides for use in citrus producing areas with endemic citrus canker (Graham and Leite, 2004; Graham et al., 2006; Graham et al., 2008).

Use of Copper and Streptomycin for Control of Citrus Canker

Copper based bactericides have been used as a standard control measure for citrus canker worldwide (Leite and Mohan, 1990). Copper is an essential metal necessary at certain levels for the normal functioning of almost all life forms, including plants, animals and microorganisms, acting as a cofactor for a number of enzymes involved in respiration and electron transport proteins. However, above certain concentrations, copper is toxic to cells mainly due to its interaction with nucleic acids, disruption of enzyme active sites, interference with the energy transport system, and ultimately, the disruption of the integrity of cell membranes (Cervantes and Gutierrez-Corona, 1994; Garcia-Horsman et al., 1994; Hegg and Burstyn, 1996). The relatively high toxicity to plant pathogens (Cha and Cooksey, 1991), low cost and low toxicity of the fixed copper compounds (Adaskaveg and Hine, 1985), and the fact that these

materials are chemically stable and are not readily washed from plants (Olson and Jones, 1983) have made their use widespread for control of bacterial diseases of foliage and fruit.

Several copper-based products have been evaluated regarding the efficiency on controlling citrus canker and other *Xanthomonas*-triggered diseases on citrus. Fixed coppers are the predominant form used in citrus growing areas with endemic citrus canker. The most efficient and well studied are copper oxychloride (Behlau et al., 2007; Graham et al. 2006; Leite et al., 1987; Medina-Urrata and Stapleton, 1985; Pereira et al., 1981), copper sulfate (McGuire, 1988; Medina-Urrata and Stapleton, 1985), copper hydroxide (Graham et al., 2006; Leite et al., 1987), cuprous oxide (Pereira et al., 1981) and copper ammonium carbonate (Gottwald and Timmer, 1995; McGuire, 1988; Timmer, 1988).

Copper ions are considered to be more toxic to microorganisms than complexed forms (Gadd and Griffiths, 1978; Menkissoglu and Lindow, 1991; Zevenhuizen et al., 1979). The concentration of copper ions on leaves depends on the equilibrium established between the complexed and soluble forms of copper (Menkissoglu and Lindow, 1991). Fixed copper compounds are predominantly insoluble on the plant surface (Menkissoglu and Lindow, 1991) and copper ions are slowly released after application. Thus, fixed coppers are less phytotoxic to plants and provide better residual activity against diseases than can be achieved with non-fixed copper. Once applied, copper particles provide a protective film that acts as a barrier that when contacted with water and low pH slowly releases copper ions that are toxic to bacterial cells (Gadd and Griffiths, 1978; Zevenhuizen et al., 1979). Exudates from the plant and microorganisms

also play an important role in copper solubility by forming weak acids that lower the pH of the water on the plant surface, which increases copper solubility and availability (Arman and Wain, 1958).

Copper bactericides have no curative or systemic activity and are usually applied preventively for citrus canker control. Such bactericides are used to reduce inoculum buildup on susceptible leaf flushes and to protect expanding fruit surfaces from infection (Timmer, 1988; McGuire, 1988; Gottwald and Timmer, 1995; Behlau et al., 2008; Graham and Leite Jr., 2004). Timing of application and effectiveness of copper-based sprays depend on several factors, such as environmental conditions, grove age, susceptibility of the citrus cultivar, and integration with other control measures (Gottwald et al., 2002; Stall and Seymour, 1983). Usually copper is applied during the spring and summer months, when climatic conditions are most favorable to the pathogen and trees are constantly producing susceptible vegetative tissue. For effective control of citrus canker, the number of sprays per season may vary from two to five (Leite and Mohan,1990; Leite et al., 1987). However, when climatic conditions for the development of the disease are highly favorable and/or the amount of susceptible plant tissue is abundant for a prolonged period, as observed for young groves, more sprays may be necessary (Leite and Mohan,1990).

Alternatively, streptomycin has been tested to complement copper sprays for control of citrus canker. Streptomycin is an antibiotic used for control of human pathogens which also is used as a pesticide to control bacteria affecting certain fruit, vegetables, seed, and ornamental crops. Streptomycin is a protein synthesis inhibitor. It prevents initiation of protein synthesis and leads to death of bacterial cells by biding to

the S12 protein of the 30S subunit of the bacterial ribosome and interfering with the binding of formyl-methionyl-tRNA to the 30S subunit (Sharma et al., 2007; Snyder and Champress, 2003). Streptomycin is also known to prevent the normal dissociation of the 70S ribosome into the 50S and 30S subunits. Thus, formation of polysomes is inhibited. The overall effect of streptomycin seems to involve distorting the ribosome so that transition from initiation of the complex (30S-mRNA-tRNA) to chain elongating ribosome is blocked. Thus, the normal sequence of translation is disrupted, the bacteria is unable to synthesize proteins vital for its cell growth and thereby fails to survive (Sharma et al., 2007; Snyder and Champress, 2003). Streptomycin also affects bacterial cells by impairing translation of mRNA, leading to the production of defective proteins (Snyder and Champress, 2003).

In agriculture, the most extensive use of streptomycin is for control of fireblight on apple and pear. In citrus, streptomycin has not been used in commercial groves for control of citrus canker. This antibiotic has been tested as a complementary measure to copper sprays (Graham et al., 2008). The purpose is to reduce the load of copper seasonally applied in citrus groves by replacing some copper applications by streptomycin or combining the two bactericides for higher effectiveness of control.

Copper Resistance in Plant Pathogenic Bacteria

The reduction in efficacy of copper sprays in controlling plant bacterial diseases has been previously reported (Adaskaveg and Hine, 1985; Cazorla et al., 2002; Martin at al., 2004; Rinaldi and Leite, 2000). Such a lack of effectiveness is mostly due to the development of bacterial strains resistant to copper. Previous studies indicate that copper resistant (Cu^R) strains have been identified in many plant pathogenic bacterial species, including *Pseudomonas* (Andersen et al., 1991; Bender and Cooksey, 1986;

Cazorlae et al., 2002; Scheck and Pscheit, 1998; Sundin et al., 1989), *Pantoea* (Nischwitz et al., 2007), *Erwinia* (Al-Daoude et al., 2009), and *Xanthomonas* (Adaskaveg and Hine, 1985; Cooksey et al., 1990; Marco and Stall, 1983; Martin et al., 2004; Ritchie and Dittapongpitch, 1991; Stall et al., 1986).

Plant pathogenic bacterial isolates obtained from regions where copper has been regularly applied for an extended period to control bacterial diseases have shown higher levels of copper resistance (Adaskaveg and Hine, 1985) and Cu^R strains are poorly controlled by standard applications of copper based compounds (Marco and Stall, 1983). The selection of copper resistant strains seems to be the major reason for control failures following management with copper bactericides (Cazorla et al., 2002). Once Cu^R strains develop, the application of copper on plants is no longer effective for disease control as resistant populations increase rapidly (Sundin et al., 1989).

Most of the genes associated with copper resistance from plant pathogenic bacteria are located on plasmids (Bender and Cooksey, 1986, 1987; Bender et al., 1990; Cazorlae et al., 2002; Cooksey, 1987; Cooksey, 1990b; Stall et al., 1986). In *P. syringae* pv. *tomato*, the copper resistance genes reside on a 35-kilobase pair plasmid in strains isolated in California (Bender and Cooksey, 1986, 1987; Cooksey, 1987). In *X. campestris* pv. *vesicatoria*, the copper resistance determinant resides on large plasmids in strains isolated in Florida and Oklahoma (Bender et al., 1990; Mellano and Cooksey, 1988a). Gene clusters associated with the chromosome also may be related to copper resistance in some bacteria such as *Pseudomonas* (Lim and Cooksey, 1993) and *Xanthomonas* (Basim et al., 2005; Lee et al., 1994). According to Basim et al. (2005), chromosomal ORF1 is essential for copper resistance and was found to play an

important role in regulation of the system for the strain XvP26 of *X. campestris* pv. *vesicatoria*. In *Escherichia coli*, additional chromosomal genes that function in copper uptake are required for resistance and apparently for normal transport and management of cellular copper (Rogers et al., 1991).

Copper sequestration and copper efflux have been suggested as the main mechanisms for copper resistance in bacteria (Cooksey, 1993). Cellular copper sequestration is the main mechanism for copper resistance in strains of *Pseudomonas syringae* (Cooksey, 1990). Colonies of Cu^R strains of *P. syringae* pv. *tomato* become blue on media amended with high levels of copper, suggesting that the bacteria accumulate this metal (Cha and Cooksey, 1991). *P. syringae* strains containing the *cop* operon accumulate more copper than strains lacking the operon (Bender and Cooksey, 1987; Cha and Cooksey, 1991; Cooksey and Azad, 1992). The *cop* operon, which is composed of the *copABCD* genes (Mellano and Cooksey, 1988a,b), is thought to confer copper resistance to *P. syringae* at least in part by sequestering and accumulating copper in the periplasm with copper-binding proteins, which may prevent toxic levels of copper from entering the cytoplasm (Cha and Cooksey, 1991; Cooksey, 1993). According to Rouch et al. (1985), genes that confer copper resistance are regulated and induced only by high levels of copper. Mills et al. (1993) demonstrated that *P. syringae* employs the *copRS* sensory transduction genes, located downstream of the *copABCD* operon, to alter gene expression in response to environmental stimuli and regulate copper resistance gene expression.

In *P. syringae*, the Cop proteins, CopA (72 kDa), CopB (39 kDa), and CopC (12kDa), are produced only under copper induction (Bender and Cooksey, 1987; Cha

and Cooksey, 1991; Mellano and Cooksey, 1988a,b). CopA and CopC are periplasmic proteins and help to prevent the entry of toxic copper ions into the cytoplasm, whereas CopB is an outer membrane protein and seems to be associated with external copper binding in the bacterial cell (Cha and Cooksey, 1991). CopD, a probable inner membrane protein, apparently functions in copper transport (Cha and Cooksey, 1991, 1993). CopC binds one atom of copper per protein molecule, while CopA binds about 11 atoms per protein. However, since the concentration of these c*op*-encoded proteins does not increase at higher levels of copper, while total accumulated copper does, their role at higher levels of copper, when their binding capacity would seem to be saturated, might be in the delivery of copper ions to other binding components of the cell wall (Cha and Cooksey, 1991). While *copA* and *copB* seem to be essential for resistance, *copC* and *copD* are required for full resistance, but some resistance can be conferred in the absence of the latter two genes (Bender and Cooksey, 1987; Mellano and Cooksey, 1988a,b). *copCD* seem to function in copper uptake, balancing the periplasmic copper sequestering activity (Cha and Cooksey, 1993).

In *E. coli,* copper resistance is regulated by different systems, including the multi-copper oxidase CueO, which protects periplasmic enzymes from copper-mediated damage (Grass and Rensing, 2001), the *cus* determinant, that confers copper and silver resistance (Munson et al., 2000) and the *pcoABCD* operon (Rensing et al., 2000). The latter is an efflux mechanism and is responsible for pumping excess copper out of the cytoplasm (Cooksey, 1993). According to Rouch et al. (1985), due to the export of copper to the outer cell environment, *E. coli* cells expressing *pco* genes accumulate less copper than wild type strains. The *pcoABCD* operon shares homology

with the *copABCD* operon of *P. syringae* and, as in *P. syringae*, is followed by two regulatory genes, *pcoR* and *pcoS*, responsible for induction of copper resistance (Brown et al., 1995; Mellano and Cooksey, 1988a). Copper inducibility of the *pco* genes of *E. coli* showed that the lag phase observed upon addition of copper to the growth medium could be reduced by pre-induction with copper sulfate (Rouch et al., 1985). *pcoE* has also been associated with the *pcoABCDRS* operon (Brown et al., 1995) however, Lee et al. (2002), demonstrated that this gene has no influence on copper resistance in *E. coli*.

Copper resistance genes have also been cloned from *Xanthomonas* species, including *X. vesicatoria* (Cooksey et at.. 1990; Garde and Bender, 1991; Basim et al., 2005), *X. arboricola* pv. *juglandis* (Lee et al., 1994) and *X. perforans* (Voloudakis et al., 2005). The plasmid-borne copper resistance determinants in *X. vesicatoria* have similarities to the *cop* operon from *P. syringae* (Voloudakis et al., 1993). However, on the chromosome the organization of the copper resistance genes appears to be uncommon in *X. vesicatoria*, and occurrence of this type of resistance is rare (Basim et al., 2005). Copper resistance genes in *X. arboricola* pv. *juglandis* are located on the chromosome and have the same general *copABCD* structure as the genes from *P. syringae*, with some differences in DNA sequence and gene size (Lee et al., 1994). In *Xanthomonas perforans* copper resistance genes are plasmid-encoded and expression of these genes was demonstrated to be regulated by *copL*, which is the immediate ORF upstream of *copAB* (Voloudakis et al., 2005). Other ORF's, namely *copM*, *copG* and *copF*, have been identified downstream of *copLAB* in *Xanthomonas perforans* (Voloudakis et al., 2005). However, the involvement of these genes in copper resistance remains unclear. The *copRS* regulatory genes, which are present in *P. syringae*

(Mellano and Cooksey, 1988a), have not been found in *Xanthomonas* (Lee et al., 1994; Voloudakis et al., 2005).

Increasing copper accumulation with exposure to increasing concentrations of copper is common to several species of copper resistant *Pseudomonas*, suggesting that they have similar resistance mechanisms involving copper sequestration (Cooksey and Azad, 1992). There are similarities between the *cop* operon from *P. syringae* and copper resistance genes from *X. campestris* (Cooksey et al., 1990; Voloudakis et al., 1993) and *E. coli* (Tetaz and Luke, 1983). Cooksey et al. (1990) reported the occurrence of plasmid and chromosomal DNA homology to the copper resistance operon of *P. syringae* pv. *tomato* in three saprophytic species of *Pseudomonas* and two plant pathogenic species, *P. cichorii* and *X. campestris* pv. *vesicatoria*. However, such homology did not confer resistance in copper sensitive strains of *P. syringae* pv. *tomato*, *P. cichorii*, and *P. flimorescens*, suggesting that these genes have some other function and may be indigenous to certain *Pseudomonas* species (Cooksey et al., 1990). The apparent lack of similarity between copper resistance genes from *P. syringae* and *X. campestris* observed in previous studies (Bender and Cooksey, 1987) and the substantial conserved nature of the 35-kb plasmid (Cooksey, 1987), initially suggested that copper resistance may have developed independently in these two species. Nevertheless, after using uniform hybridization conditions Voloudakis et al. (1993) found a close relation between copper resistance genes from *X. campestris* pv. *vesicatoria* and the *cop* operon from *P. syringae*. Thus, the exchange of plasmid DNA between these two species, or other bacteria, is a more plausible explanation for the observed similarities between *cop* and copper resistance genes in xanthomonads.

The frequency of copper resistant bacterial strains may be enhanced by conjugation (Sundin et al. 1989; Stall at al., 1986; Tetaz and Luke, 1983). Plasmid transfer of antibiotic and copper resistance has been previously reported for *P. syringae* (Bender and Cooksey, 1986). Two conjugative plasmids of *P. syringae* pv. *tomato* PT23 are involved in the copper resistance phenotype (Bender and Cooksey, 1986). One of them, pPT23A, has a similar size to the conjugative copper plasmid identified previously in *E. coli* (Tetaz and Luke, 1983), however it is smaller than the plasmid identified in *X. campestris* pv *vesicatoria* (Bender et al. 1990). Copper sensitive strains of *P. syringae* pv. *syringae* isolated from cherry trees were able to acquire the 61 kb plasmid containing genes which confer copper resistance from all donors tested, and the transfer frequency was highest between isolates from the same orchard (Sundin et al. 1989).

The possibility for plasmid transfer between *X. campestris* pathovars exists on both host and nonhost plants (Bender et al., 1990). According to Timmer et al. (1987), *X. campestris* pv. *alfalfae, campestris, translucens*, and *pruni* can multiply on tomato leaves under conditions of high relative humidity. Moreover, *X. campestris* pv. *vesicatoria* populations were able to multiply on the leaves of nonhost plants such as plum and peach. Once the right conditions are provided and bacteria are present, interpathovar transfer of copper resistance plasmid may occur in nature (Bender et al., 1990). A highly conjugative copper resistance plasmid from X*. campestris* pv. *campestris* hybridized strongly with the cloned copper resistance genes from *X. campestris* pv. *vesicatoria* (Voloudakis et al., 1993). This observation points out the probability that copper resistance plasmids have been exchanged among pathovars of

related plant pathogens, as suggested for *P. syringae* (Bender and Cooksey, 1986; Cooksey, 1990).

The development of Cu^R strains of Xcc has been reported only in Argentina (Canteros, 1996). The resistant strains were first isolated in 1994 from a citrus grove located in the province of Corrientes which showed a lack of response to the numerous copper sprays used for control of recurrent outbreaks of citrus canker (Canteros, 1996). Since then, according to Canteros et al. (2008), Cu^R strains have not spread within the Xcc population in the citrus growing areas in Argentina. A recent survey showed that the resistance is presently occurring in a few different locations in Corrientes and in an isolated grove in the province of Formosa, northwest of Corrientes (Canteros, 2008).

Streptomycin Resistance in Plant Pathogenic Bacteria

Other contact bactericides including antibiotics have not been as effective as copper for controlling citrus canker (Leite and Mohan, 1990; Leite et al., 1987; McGuire, 1988; Timmer, 1988). Additionally, antibiotic resistance has developed within various plant pathogen populations (Burr et al., 1988; De Boer, 1980; Schroth et al., 1979; Stall and Thayer, 1962). Development of resistance to streptomycin in plant pathogens and in other plant-associated bacteria seems to be relatively common and resistance to this antibiotic has been reported in the phytopathogens *Erwinia amylovora* (Chiou and Jones, 1991; Loper et al., 1991; Schroth et al., 1979), *P. syringae* (Burr et al., 1988, DeBoer, 1980; Jones et al., 1991), and *X. campestris* pv. *vesicatoria* (Minsavage et al., 1990, Stall and Thayer, 1962). Good control of bacterial spot on tomato caused by *X. campestris* pv. *vesicatoria* was obtained with streptomycin sprays in the early stages of the crop; however, later in the season, there was no significant control of the disease by this antibiotic (Stall and Thayer, 1962). Furthermore, strains isolated early in the season

were more susceptible to the antibiotic than strains isolated later in the season, when streptomycin was visually ineffective (Stall and Thayer, 1962). The development of resistance to streptomycin in Xcc populations affecting citrus has not been reported yet and this is most likely due to the fact that this antibiotic has not been used for control of citrus canker in commercial groves.

Streptomycin resistance has been shown to be associated with *strA-strB* genes (Chiou and Jones, 1995; Huang and Burr, 1999) carried on a conjugative plasmid (Burr et al., 1988; Huang and Burr, 1999; Norelli et al., 1991). However, there is evidence to support that chromosomal-mediated resistance (Schroth et al., 1979) is also involved in streptomycin resistance in plant-pathogenic bacteria. Thus, the mechanisms for streptomycin resistance are related to a chromosomal mutation that results in the alteration of the ribosomal protein S12 which is the target site for binding of streptomycin on bacterial ribosomes (Chang and Flaks, 1972) or to resistant plasmids that carry determinants homologous to *strA-strB* genes, which encode streptomycin modifying enzymes, preventing it from binding to the bacterial ribosome (Scholz et al. 1989). Modification of the target molecule results from a point mutation in the highly conserved gene *rpsL* which codes for the protein S12. Such a mutation makes bacteria resistant to extremely high levels of streptomycin, but the resistance cannot be easily transferred to other bacteria. It is usually transferred only during bacterial division. Bacteria that are able to enzymatically inactivate streptomycin have usually acquired this capability through the acquisition of *strA-strB* genes, which code for the enzymes necessary to inactivate streptomycin. These genes are carried by genetic elements, such as plasmids or transposons, which can be transferred and can confer resistance to

other bacteria including bacteria from other species or other genera. Those bacteria are resistant to lower levels of streptomycin than bacteria that have a mutation in the *rpsL* gene (McManus et al., 2002). Different mutation sites in *rpsL* gene (Chiou and Jones, 1995) and ribotype fingerprints (McManus and Jones, 1995) have been observed for Sm^R mutants of *E. amylovora* from different regions of the world, indicating that that resistance developed independently and has been selected for multiple times.

Hybridization analyses indicated that a homologous streptomycin resistant determinant has been detected in several phytopathogenic bacterial populations, including *E. amylovora*, *P. syringae* pv. *papulans*, *P. syringae* pv. *syringae*, and *X. campestris* pv. *vesicatoria* (Chiou and Jones, 1993; Minsavage et al., 1990; Sundin and Bender, 1993). The *strA-strB* genes in *P. syringae* and *X. campestris* were encoded on elements closely related to transposon Tn5393 previously reported in *E. amylovora* (Chiou and Jones, 1993), designated Tn5393a and Tn5393b, respectively (Sundin and Bender, 1995). The dissemination of Tn5393 and derivatives in phytopathogenic prokaryotes confirms the importance of these bacteria as reservoirs of antibiotic resistance in the environment (Chiou and Jones, 1993; Sundin and Bender, 1995).

X. campestris pv. *vesicatoria* strains with different levels of streptomycin resistance were pathogenic to tomato plants, suggesting that resistance to this antibiotic is not related to pathogenicity (Stall and Thayer, 1962). Apparently, the mechanism involved in development of streptomycin resistance is the selection of resistant strains rather than promoting adaptive change in the bacteria in the presence of the bactericide (Stall and Thayer, 1962; Sundin and Bender, 1995). Furthermore, evidence suggests that streptomycin resistance may be linked to copper resistance (Ritchie and

Dittapongpitch, 1991; Sundin and Bender, 1993). According to Sundin and Bender (1993), the *P. syringae* pv. *syringae* population developed resistance to one or both of these compounds on conjugative plasmids in response to the selection pressure of copper and streptomycin bactericidal sprays. For Ritchie and Dittapongpitch (1991), all streptomycin resistant strains of *Xanthomonas campestris* pv. *vesicatoria* were also copper resistant; conversely, no copper-sensitive strains showed streptomycin resistance.

Project Goal and Objectives

The goal of this project was to assess the risks for the development of copper and streptomycin resistant strains of Xcc. The objectives were: (i) survey for copper resistant strains of Xcc in Florida and Brazil and *Xanthomonas alfafae* subsp. *citrumelonis* (Xac) in Florida; (ii) monitor for the presence of resistant populations of Xcc and epiphytic bacteria on young citrus trees treated with copper or streptomycin; (iii) screen bacteria from the citrus phyllosphere for copper resistance genes; (iv) analyze the possibility of horizontal transfer of copper and streptomycin resistance genes; (v) clone and characterize copper resistance determinants in Xcc and Xac; and (vi) compare copper resistance determinants from Xcc to other bacteria.

CHAPTER 2
SURVEY FOR COPPER RESISTANT STRAINS OF *Xanthomonas citri* subsp. *citri* IN FLORIDA AND BRAZIL AND *Xanthomonas alfalfae* subsp*. citrumelonis* IN FLORIDA

Introduction

One of the greatest concerns surrounding the use of copper based bactericides for control of citrus canker is that numerous sprays per season are usually necessary for efficacious disease control and frequent use of copper may lead to development of resistant strains of the pathogen as reported previously for *Xanthomonas* (Adaskaveg and Hine, 1985; Cooksey et al., 1990; Marco and Stall, 1983; Martin et al., 2004; Ritchie and Dittapongpitch, 1991; Stall et al., 1986) and *Pseudomonas* (Andersen et al., 1991; Bender and Cooksey, 1986; Cazorla et al., 2002; Scheck and Pscheit, 1998; Sundin et al., 1989) affecting different crops.

Copper resistant (Cu^R) strains of *Xanthomonas citri* subsp. *citri* (Xcc) (syn. *Xanthomonas axonopodis* pv. *citri*), the causal agent of citrus canker, have been reported only in Argentina (Canteros, 1996). The resistant strains were first isolated in 1994 from a citrus grove located in the province of Corrientes which showed a lack of response to the numerous copper sprays used for control of recurrent outbreaks of citrus canker (Canteros, 1996). Since then, according to Canteros et al. (2008), Cu^R strains have not spread within the Xcc population in the citrus growing areas in Argentina. A recent survey showed that the resistance is presently occurring in a few different locations in Corrientes and in an isolated grove in the province of Formosa, northwest of Corrientes (Canteros, 2008).

Florida and Brazil have the largest citrus growing areas in the world and together are responsible for more than 90% of the world's production of concentrated orange juice (Agrianual, 2009; Florida Citrus Mutual, 2010). In Florida, the citrus canker

eradication program was halted in 2005 after an unsuccessful attempt to control the spread of the disease throughout the state (Gottwald and Irey, 2007). Since then, copper sprays have been incorporated as one of the most important strategies for managing the disease in Florida (Graham et al, 2006). In Brazil, the citrus industry is concentrated in São Paulo, which is the only state where citrus canker eradication is still practiced in that country (Barbosa et al., 2001; Massari and Belasque Jr., 2006; São Paulo, 1999;) and copper sprays are not regularly used for control of this disease. In contrast, in Paraná state, located in southern Brazil and bordered on the north by São Paulo state, citrus canker has been endemic for more than 20 years and copper bactericides have been widely used during this time as one of the main strategies of control (Leite and Mohan, 1990). Nevertheless, there is a concern that long-term use of copper based bactericides for managing citrus canker in Florida and Brazil may lead to development of Cu^R strains of Xcc, which can potentially reduce the efficacy of copper against the pathogen as was observed in Argentina (Canteros, 1996) and constrain the limited set of measures available for controlling the disease (Graham et al., 2008).

Citrus bacterial spot (CBS) caused by *Xanthomonas alfalfae* subsp. *citrumelonis* (Xac) (syn. *X. campestris* pv. *citrumelo*, *X. campestris* pv. *citri* strain E) is another disease caused by xanthomonads on citrus in Florida. CBS has quite distinct symptoms compared to citrus canker and the disease has only been reported in Florida nurseries. Although CBS has been important in outdoor nurseries, new regulations in Florida mandate the propagation of certified, pathogen free citrus trees in enclosed nurseries protected from pathogens and their insect vectors (FDACS-DPI, 2010). Thus, since CBS is restricted to nursery environments, the disease is not a threat to production

citrus (Graham and Gottwald, 1991). However, for several years, Xac populations in nurseries were exposed to frequent sprays of copper as a protective measure against CBS-induced bud failure (Graham et al, 1999) and other foliar diseases affecting citrus nursery plants. Considering that Xcc and Xac are two very closely related *Xanthomonas* species and Cu^R genes are mostly harbored on conjugative plasmids which have been shown to move among different species of *Xanthomonas* as seen in Chapter 3, the concern in using copper for control of CBS is that if copper resistance has developed in strains of Xac, resistance could then move horizontally to Xcc in Florida.

The objective of this study was to investigate whether copper resistance has been transferred to Xcc and Xac strains in Florida where the use of copper sprays for control of citrus canker is a recent practice in commercial citrus fields but it has a long history of use in nurseries for control of CBS and in Paraná State, Brazil, where copper has been applied to citrus groves for more than two decades.

Material and Methods

Florida

The presence of Cu^R in *Xanthomonas* affecting citrus in Florida was assessed by screening strains from the collection of plant bacterial strains of the Florida Department of Agriculture and Consumer Services, Division of Plant Industry (DPI), Gainesville, FL. Overall, 356 strains of Xcc isolated throughout the citrus growing area from 1997 to 2007 (Table 2-1), 28 Xcc strains isolated from citrus nurseries from 2007 to 2009 (Table 2-1) and 54 Xac strains obtained from citrus groves and nurseries around the state between 1999 and 2009 were evaluated (Table 2-2). The strains were single colony subcultured on nutrient agar (NA) from glycerol vials stored at -80^{o}C. For screening, cultures were grown overnight on NA amended with 20 mg L^{-1} of copper for induction of

resistance (Basim et al., 2005), suspended in tap water to an approximate concentration of 10^8 cfu mL^{-1} and then spotted in duplicate (10 µL per spot) on MGY agar (mannitol-glutamate yeast agar) (Cooksey and Azad, 1992) amended with 200 mg L^{-1} of copper (Basim et al., 2005). Inoculated plates were incubated at 28^{o}C for 96 h and then assessed for bacterial growth. Cu^R A44 and copper sensitive (Cu^S) 306 strains of Xcc were used as positive and negative controls, respectively. Copper was added to the medium as copper sulfate pentahydrate ($CuSO_4 \cdot 5H_2O$) from a 50 mg mL^{-1} stock solution before autoclaving. Strains that developed confluent growth on MGY agar amended with 200 mg L^{-1} of copper after 96 h of incubation were rated as Cu^R.

Brazil

In Brazil, screening of Cu^R strains of Xcc was conducted for strains isolated from citrus in Paraná State, where citrus canker has been endemic for more than 20 years and copper based bactericides have been frequently sprayed for control of the disease during this period. Assessments were performed by evaluating pure strains of the pathogen and also by assessing washings of canker-symptomatic citrus leaves collected from citrus groves treated regularly with copper. Xcc cultures were obtained from the collection of plant pathogenic bacteria of the laboratory of Bacteriology and Virology of the Agronomic Institute of Paraná (IAPAR), Londrina, Paraná, Brazil, where this part of the study was conducted.

Forty strains of Xcc isolated in 1996 and 1997 from different regions of the citrus growing area in Paraná state (Table 2-3) were assessed on MGY agar amended with 200 mg L^{-1} of copper (Basim et al., 2005). Before challenging on copper amended MGY, strains were grown overnight on NA amended with 20 mg L^{-1} of copper for induction of resistance (Basim et al., 2005). Following overnight incubation, bacterial

suspensions were prepared in sterile tap water and adjusted to approximately 5 x 10^8 cfu mL^{-1} using a spectrophotometer (absorbance 0.3 at 600 nm). Aliquots of 10 µL of bacterial suspensions were spotted in triplicate on copper-amended MGY agar for resistance assessment. Plates were incubated at 28 ^oC for 96 h before assessing growth. Cu^R *X. euvesicatoria* 81-23 and Cu^S Xcc 306 strains were used as positive and negative controls, respectively. MGY media was amended with copper as copper sulfate pentahydrate ($CuSO_4 \cdot 5H_2O$) which was added to the medium from a 50 mg mL^{-1} stock solution before autoclaving. Strains that produced confluent growth on MGY agar amended with 200 mg L^{-1} of copper after incubating for 96 h were rated as Cu^R.

To assess for the presence of Cu^R Xcc strains in the field, 46 samples of canker-symptomatic leaves were collected from groves of the major citrus-growing area in Paraná located in the municipalities of Paranavaí (43 samples) and São João do Caiuá (3 samples). Each sample was composed of 10 to 20 leaves randomly collected within the block. Citrus leaf washings were prepared in Erlenmeyer flasks by adding 10 mL of MGY broth per gram of leaf. MGY broth was amended with 1 mg L^{-1} of copper and 1% peptone to induce resistance of presumptive Cu^R strains of Xcc prior to plating on a higher concentration of copper and to help release bacterial cells from the leaves into the broth, respectively. Flasks were shaken vigorously for 2 h at room temperature (RT) using a wrist shaker. Washing aliquots were plated on MGY-KCH supplemented with 75 mg L^{-1} of copper (see Chapter 3 for more details) using 100 µL of 10^{-1} to 10^{-3} dilutions per plate. Plates were incubated for 96 h at 28^oC before assessment of bacterial growth.

Results

While the Cu^R positive control of Xcc grew overnight on MGY agar amended with 200 mg L^{-1} of copper, none of the Xcc strains isolated from citrus groves in Paraná and Florida were able to grow on copper-amended MGY agar after 4 days of incubation (Figure 2-1). Likewise, no Cu^R strain of Xcc were recovered on the semi-selective medium MGY-KCH amended with copper from assays of washings of canker-symptomatic leaves recently collected from citrus groves in Paraná.

In contrast to Xac strains, numerous strains of Xac isolated from Florida nurseries were identified as Cu^R. Thirty-one of 54 Xac strains screened (57%) grew overnight on MGY agar + 200 mg L^{-1} of copper (Figure 2-1). Cu^R strains of Xac were detected in isolates from 1999 to 2009,all of the years that nursery strains were screened, and were present in 14 of the 20 counties surveyed , including Broward, Clay, Collier, Glades, Hendry, Hernando, Highlands, Hillsborough, Lake, Lee, Manatee, Miami-Dade, Saint Lucie, and Polk (Table 2-4).

Discussion

Several genes are involved in copper resistance in bacteria and spontaneous mutations are unlikely to confer resistance as commonly observed for several antibiotics. Copper resistance in bacteria is acquired through horizontal transfer of plasmids or transposable elements carrying the copper resistance genes (Chiou and Jones, 1991, 1995; Han et al., 2003; McManus and Jones, 1994; Sundin and Bender, 1996). This is a process commonly used by bacteria to exchange genetic material in the environment and promptly adapt to adverse and changing local conditions (Lilley and Bailey, 1997; Lilley et al., 1994). The development of copper resistance in bacteria is highly dependent on the selection pressure of copper exposure on the bacterial

population. Thus, the frequent use of copper sprays for control of bacterial diseases affecting crops may lead to the development of Cu^R strains (Adaskaveg and Hine, 1985; Andersen et al., 1991; Bender and Cooksey, 1986; Cazorla et al., 2002; Cooksey et al., 1990; Marco and Stall, 1983; Martin et al., 2004; Ritchie and Dittapongpitch, 1991; Scheck and Pscheit, 1998; Stall et al., 1986; Sundin et al., 1989).

Florida and Paraná State, Brazil are currently under a contrasting scenario regarding the amount of copper that the citrus growing areas in these two regions have been exposed to in the last years. In Florida, frequent use of copper sprays for control of canker has been adopted just recently, after the citrus canker eradication program was suspended in 2005, but Xac have been exposed to copper for years to control CBS in nurseries. In contrast, Paraná citrus groves have been sprayed with copper based bactericides for control of citrus canker for more than two decades. In Argentina, copper sprays had been used for a similar period in citrus growing areas with citrus canker and Cu^R strains of Xcc have been discovered (Canteros, 1996). In our survey for copper resistance none of the selected strains of Xcc from Florida and Brazil were identified as Cu^R. The strains from Brazil were isolated in 1996-1997, just a few years after the eradication program was replaced by an integrated management approach for citrus canker that includes, among other measures, the use of copper sprays to protect the foliage and fruit from damage (Leite and Mohan, 1990). Likewise, Xcc populations in Florida have not been exposed to copper for a prolonged period. Moreover, samples of leaves with citrus canker collected in 2009 and 2010 from groves in Paraná did not reveal the presence of Cu^R strains in that area. Although this indicates that copper resistance in Xcc has either not yet developed or has not spread in the citrus growing

areas of Parana or Florida, constant surveillance is advisable to assess the risk of copper resistance as long as copper sprays are repeatedly used in citrus groves with endemic canker.

The majority of the Xac strains screened in this study were identified as Cu^R. Although strains isolated from CBS in nurseries as early as 1999 were found to be resistant, this is the first time copper resistance has been reported for Xac. The resistant strains were isolated from 14 counties throughout the state indicating that the resistance is widely spread. Most likely, copper resistance has developed in Xac because citrus nurseries have been frequently sprayed with copper bactericides for control of CBS from the time of the eradication program in 1984 (Graham and Gottwald 1991). Dissemination of Cu^R Xac throughout Florida has also likely occurred due to the distribution and use of infected or contaminated budwood for citrus propagation in addition to the copious application of copper that promoted and maintained the selection of resistant strains statewide.

Conditions for the development of CBS in the field are not as favorable as in nurseries. Although incidence of this disease in groves is uncommon it can still be found in citrus producing areas in Florida. For most of the Cu^R strains of Xac identified there is no information on whether they were isolated from citrus nurseries or groves. However, three of the strains identified as Cu^R, 1347, 1381 and 1382 from Collier County, are known to have been isolated from groves. Since Xac and Xcc share the same host and thrive under similar environmental conditions, the concern is that the interaction between these two bacteria in the mesophyll of leaves on newly planted nursery trees

coinfected with CBS and citrus canker in citrus groves could result in horizontal transfer of Cu^R from Xac to Xcc.

Table 2-1. Geographical distribution of *Xanthomonas citri* subsp. *citri* strains from Florida screened for copper resistance.

Year of isolation	County of origin (No. of strains tested)	Total strains tested
Xanthomonas citri subsp. *citri* isolated from citrus groves		
1997	Manatee (4)	4
1998	Indian River (1), Manatee (1)	2
1999	Collier (2), Hendry (1)	3
2000	Hendry (6), Manatee (1)	7
2001	DeSoto (4), Collier (3), Hendry (2), Indian River (1)	10
2002	Orange(3), Okeechobee (2), Highlands (2), Hendry (1), Hardee (1), Brevard (1)	10
2003	Highlands (3), DeSoto (3), Lee (2), Orange (1), Manatee (1)	10
2004	Highlands (6), DeSoto (1)	10
2005	DeSoto (15), Hendry (14), Charlotte (13), Saint Lucie (12), Hardee (8), Hillsborough (7), Highlands (6), Polk (6), Indian River (4), Manatee (3), Martin (3), Collier (2), Glades (2), Lee (2), Okeechobee (2), Osceola (1)	100
2006	DeSoto (18), Hendry (14), Hardee (11), Indian River (10), Saint Lucie (9), Charlotte (6), Collier (6), Glades (6), Highlands (6), Martin (6), Polk (3), Manatee (2), Pinellas (1), Brevard (1), Lee (1)	100
2007	Saint Lucie (22), Hendry (16), Hardee (14), DeSoto (10), Highlands (8), Polk (7), Indian River (6), Manatee (5), Martin (5), Charlotte (2), Collier (2), Osceola (2), Glades (1)	100
Xanthomonas citri subsp. *citri* isolated from citrus nurseries		
2007	Miami-Dade (6), Polk (4)	10
2008	Miami-Dade (2), Palm Beach (2), Manatee (1), Polk (1)	6
2009	Lake (6), Duval (2), Alachua (1), Broward (1), Miami-Dade (1), Nassau (1)	12

Table 2-2. Geographical distribution of *Xanthomonas alfalfae* subsp. *citrumelonis* strains from Florida screened for copper resistance.

Year of isolation	County of origin (No. of strains tested)	Total strains tested
1999	Collier (1); Hillsborough (1); Lake (1)	3
2000	Collier (5); Palm Beach (2); Highlands (1); Hillsborough (1); Manatee (1)	10
2001	Broward (1); Hillsborough (1); Marion (1)	
2004	Highlands (2); Saint Lucie (2); Hillsborough (1)	5
2005	Saint Lucie (4); Hendry (2); Orange (1); Clay (1); Collier (1); Lee (1)	10
2006	DeSoto (3); Charlotte (2); Broward (1); Hernando (1); Hillsborough (1); Polk (1); Saint Lucie (1)	
2007	Polk (2); Collier (1); Broward (1); Glades (1); Hendry (1); Hillsborough (1); Miami-Dade (1)	9
2008	Lee (1)	1
2009	Lake (2); Miami-Dade (1); Volusia (1)	4

Table 2-3. Geographical distribution of *Xanthomonas citri* subsp. *citri* strains from Paraná State, Brazil, screened for copper resistance.

Year of isolation	Municipality of origin (No. of strains tested)	Total strains tested
1996	Alto Paraná (1)	19
	Loanda (1)	
	Marilena (1)	
	Mirador (1)	
	Nova Aliança do Ivaí (1)	
	Nova Londrina (2)	
	Paraíso do Norte (2)	
	Paranavaí (7)	
	Santa Cruz do Monte Castelo (1)	
	São Carlos do Ivaí (1)	
	São João do Caiuá (1)	
1997	Alto Paraná (1)	21
	Amaporã (1)	
	Ângulo (2)	
	Guairaçá (1)	
	Paranacity (1)	
	Paranavaí (8)	
	Planaltina do Paraná (1)	
	Querência do Norte (1)	
	Santa Isabel do Ivaí (4)	
	São Pedro do Paraná (1)	

Table 2-4. *Xanthomonas alfalfae* subsp. *citrumelonis* strains from Florida resistant to copper.

Year of isolation	Strain	County of origin
1999	1618	Hillsborough
	1672	Lake
2000	1347	Collier
	1381	Collier
	1382	Collier
	1383	Collier
	1494	Collier
	1620	Manatee
2001	1888	Hillsborough
	1902	Broward
2004	6301	Highlands
	6309	Saint Lucie
	6310	Highlands
2005	6575	Saint Lucie
	6666	Hendry
	6677	Hendry
	6739	Collier
	6922	Lee
	7252	Clay
	7833	Saint Lucie
2006	8320	Saint Lucie
	8393	Hernando
	8666	Broward
	8761	Polk
2007	7589	Glades
	8985	Miami-Dade
	9226	Broward
	9440	Collier
	9606	Hendry
2008	18410	Lee
2009	29354	Miami-Dade

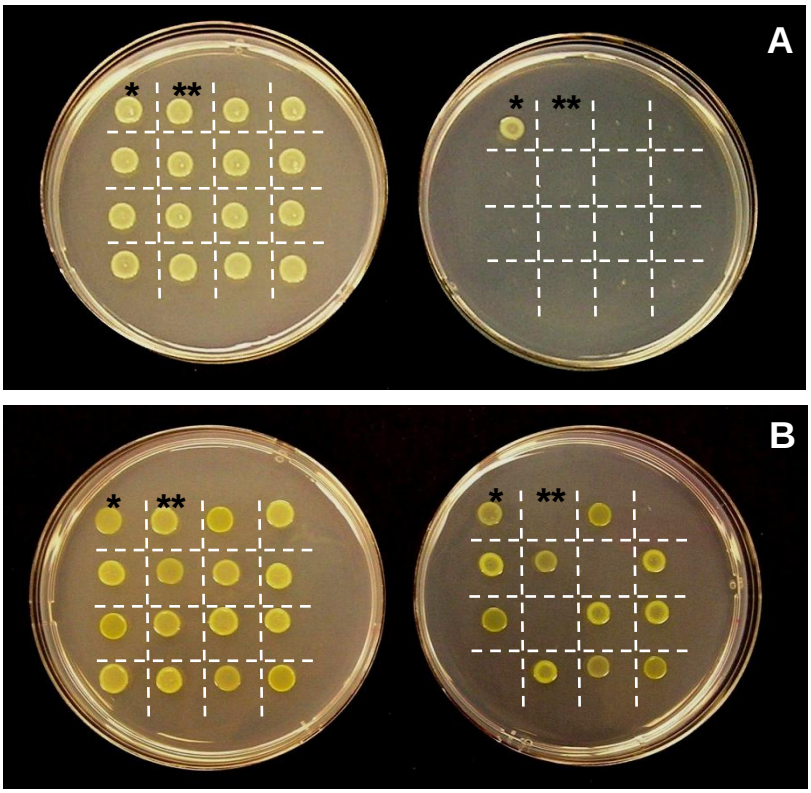

Figure 2-1. Growth of different strains of *Xanthomonas citri* subsp. *citri* (Xcc) and *Xanthomonas alfalfae* subsp. *citrumelonis* (Xac) from Florida on copper amended medium 24 h after plating. A) Xcc on mannitol-glutamate yeast extract (MGY) agar amended (right) or not (left) with copper and B) Xac on MGY agar amended (right) or not (left) with copper as copper sulfate pentahydrate ($CuSO_4 \cdot 5H_2O$) at 200 mg L^{-1}. * Copper resistant positive control strains Xcc A44 (A) and Xac 1381(B); ** copper sensitive negative control strains Xcc 306 (A) and Xac 1390 (B).

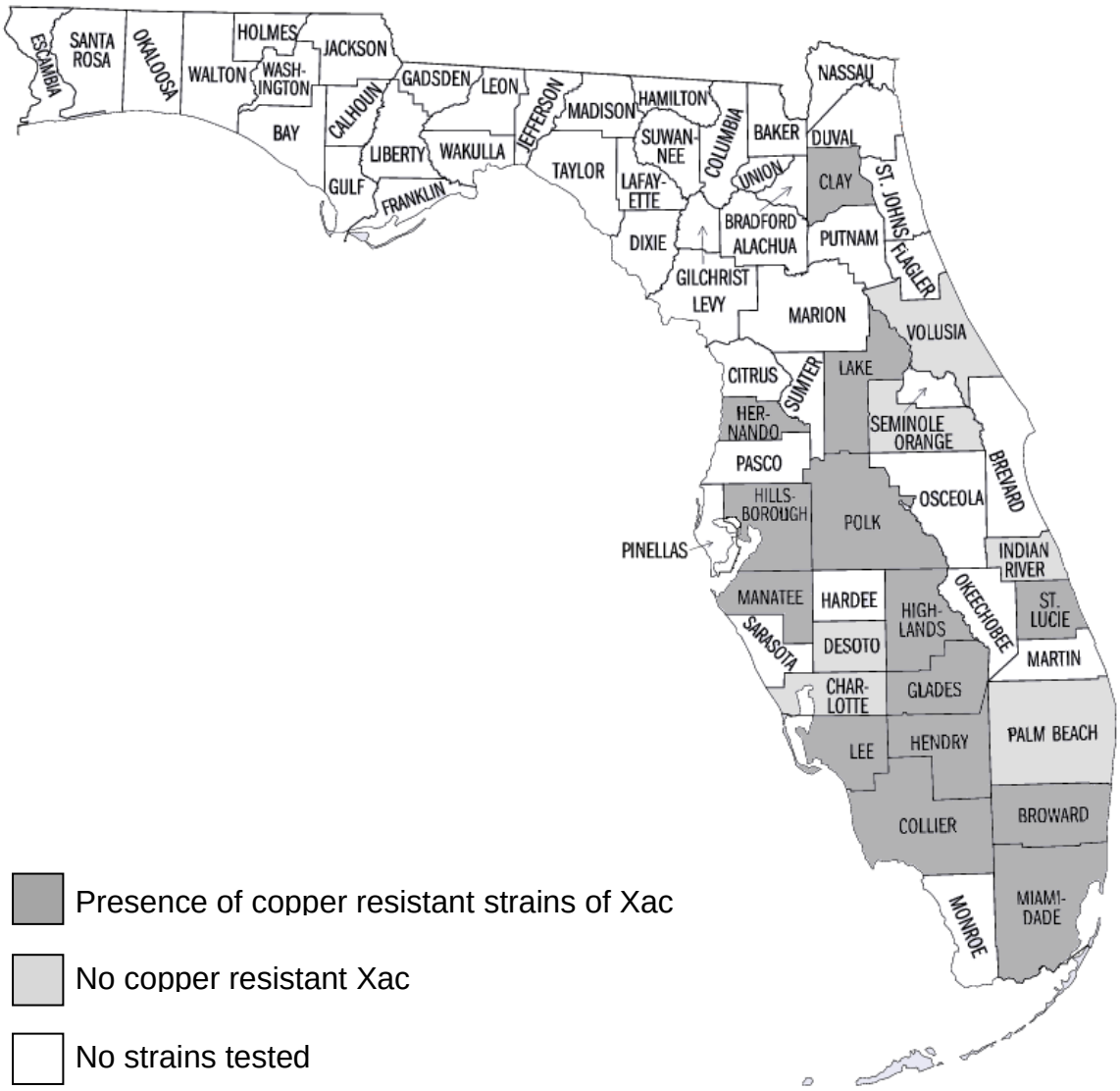

Figure 2-2. Distribution of copper resistant strains of *Xanthomonas alfalfae* subsp. *citrumelonis* (Xac) in Florida.

CHAPTER 3
RISK ASSESSMENT OF COPPER AND STREPTOMYCIN RESISTANCE DEVELOPMENT IN *Xanthomonas citri* subsp*. citri*

Introduction

Since Florida's citrus canker eradication program was halted in 2006 attention has focused on management strategies that include the use of bactericides such as copper and streptomycin for control of this disease. A concern is that widespread use of these chemicals in citrus growing areas may lead to development of resistance in strains of *Xanthomonas citri* subsp. *citri* (Xcc), the causal bacterium of citrus canker.

Copper and streptomycin resistance in bacteria develop through various processes. Because copper resistance in bacteria is regulated by several genes, the probability of spontaneous development of copper resistant (Cu^R) mutants within a bacterial population is unlikely to occur. On the other hand, spontaneous mutants could occur in the case of streptomycin resistance (Snyder and Champness, 2003). Streptomycin has been shown to interact directly with the small ribosomal subunit (Carter et al., 2000). A single mutation in the target site prevents streptomycin from binding, rendering the bacterium resistant to this antibiotic (Gale et al., 1981; Springer et al., 2001). Alternatively, streptomycin resistance may develop by horizontal gene transfer through conjugation of plasmids or transposable elements (Bender, 1996; Burr et al., 1988; Chiou and Jones, 1991, 1995; Gale et al., 1981; Han et al., 2003; McManus and Jones, 1994), which is also the primary mechanism for acquisition of copper resistance by bacteria (Voloudakis et al., 1993; Cooksey et al., 1990).

Genetic exchange of plasmids by conjugal transfer has been observed in different environments (Bjorklof et al., 2000; Canteros et al., 1995; Goodman et al., 1993; Kroer et al. 1998; Lilley et al., 1994; Sandaa and Enger, 1994; Sorensen, 1993; Weinberg and

Stotzky, 1972) and is considered to be an important process in the selective adaptation of microorganisms to shifting and challenging local environmental conditions (Lilley and Bailey, 1997). Plant surfaces are colonized by numerous and diverse bacterial species. Under favorable environmental conditions, such as high relative humidity or free water, bacterial population on leaves can reach 10^5 to 10^7 cfu per g of leaf (Hirano and Upper, 1990; O'Brien and Lindow. 1989). Bacterial communities living in the phyllosphere are in constant and dynamic interaction. These epiphytic bacteria harbor diverse plasmids which potentially increase gene exchange in these communities (Canteros et al., 1995; Lilley and Bailey, 1997; Sundin, and Bender, 1994; Sundin et al., 1994; Vivian et al., 2001) and make the phylloplane a microenvironment favorable for horizontal dissemination of genetic material (Lindow and Leveau, 2002).

The frequency of copper and streptomycin resistance genes is correlated with increasing loads of the selective chemical agents in the environment (Beining et al., 1996; Burr et al., 1988; Huang and Burr, 1999; Norelli et al., 1991; Sobiczewski et al., 1991; Stall and Thayer, 1962). Genetic horizontal transfer is a process highly dependent on bacterial populations (Levin et al., 1979; Normander et al., 1998). The higher the frequency of resistant bacteria to these chemicals in a given environment, the higher is the probability for horizontal transfer of resistance genes to sensitive bacterial strains. Thus, the expectation is that periodic application of copper or streptomycin based bactericides on crops to control bacterial diseases increases the selection pressure for the development of epiphytic bacterial populations resistant to these chemicals, elevating the risks for development of resistance within the plant pathogenic bacterial population. Once resistance genes are acquired by the plant pathogen targeted by

these chemicals, either by mutation or conjugation, the frequency of the resistant strains in the pathogen population will increase and further applications will be gradually be less effective for disease control.

The objectives of this study are: 1) to assess the risks for the development of copper or streptomycin resistance in Xcc by monitoring the resistance levels in Xcc and epiphytic bacterial populations on citrus trees repeatedly sprayed with these chemicals for control of citrus canker, and 2) to identify factors that enable the development of resistance including the presence of Xcc homologues for resistance genes in citrus epiphytic bacteria and their potential for horizontal transfer within different *Xanthomonas* species and from citrus epiphytic bacteria to *Xanthomonas*.

Material and Methods

Development of a Semi-Selective Medium for the Isolation of Copper and Streptomycin Resistant Strains of *Xanthomonas citri* subsp. *citri* from Plant Material

A series of tests were conducted to develop a semi-selective medium for the recovery of Cu^R or Sm^R Xcc from plant material (Table 2-1). The experiments aimed at suppressing contaminants to enhance growth of Xcc on various media amended with antibiotics/fungicides and the major selective components, copper or streptomycin. Initially, NGA and MGY agar were tested as basic media to be amended with selective components. NGA agar (nutrient glucose agar - nutrient agar 23.0 g L^{-1}, glucose 0.1 g L^{-1}) amended with kasugamycin (K, 16 mg L^{-1}), cephalexin (C, 16 mg L^{-1}) and chlorothalonil - BRAVO 720 (B, 12 mg L^{-1}) has been previously used as a semi-selective medium to isolate Xcc and Xac from diseased leaves (Graham and Gottwald, 1990; Roistacher and Civerolo, 1989). MGY agar (mannitol-glutamate yeast extract agar, mannitol 10 g L^{-1}, L-glutamic acid 2 g L^{-1}, KH_2PO_4 0.5 g L^{-1}, NaCl 0.2 g L^{-1},

$MgSO_4.7H_2O$ g L^{-1}, yeast extract 1.0 g L^{-1}, agar 15 g L^{-1}) is a standard medium utilized to assess copper resistance in vitro (Bender et al., 1990; Cooksey and Azad, 1992). Other adjustments consisted of assessing antibiotics and fungicides in different combinations and concentrations, evaluating growth of different Cu^R or Sm^R strains of Xcc and other species of *Xanthomonas*, and determining the optimal concentration of copper and streptomycin for amendment of the semi-selective medium to permit confluent growth of Cu^R or Sm^R Xcc and suppression of sensitive Xcc by the other selective components (Table 2-1).

Xcc strains 306, A44 and 306S were used as controls of copper/streptomycin sensitivity, copper resistance, and streptomycin resistance, respectively. Copper was used as copper sulfate pentahydrate ($CuSO_4.5H_2O$) and added to the medium from a 50 mg mL^{-1} stock solution before autoclaving. Plating of pure culture of Xcc was used to determine the recovery efficiency of Xcc on amended versus non-amended medium. Xcc was pre-grown overnight on NA (Nutrient Agar) amended with 20 mg L^{-1} of copper for induction of resistance (Basim et al., 2005), suspended in sterile tap water, and plated on the selective medium using 100 µL of suspensions at 10^6, 10^4 and 10^2 cfu mL^{-1} to assess growth of individual colonies of Xcc. Bacterial cell suspensions were adjusted in a spectrophotometer (Spectronic 20, Baush & Lomb, Inc.) to an OD of 0.3 at 600 nm, corresponding to approximately 5 x 10^8 cfu mL^{-1}, and then diluted in sterile tap water before plating. Whenever used (Table 2-1), washings from asymptomatic citrus leaves from commercial groves or washings spiked with Cu^R Xcc were plated on the medium to evaluate the efficiency of antibiotics and fungicides for suppressing growth of non-target microorganisms present in the citrus phyllosphere in the presence and

absence of Cu^R Xcc. Citrus leaf washings were prepared in Erlenmeyer flasks by adding 10 mL of MGY broth per gram of leaf. MGY broth was amended with 1 mg L^{-1} of copper and 1% peptone to induce presumptive copper resistance genes present in the bacterial population prior to plating on a higher concentration of copper and to help release bacterial cells from the leaves into the broth, respectively. Flasks were shaken vigorously for 2 h at room temperature (RT) using a wrist shaker (Burrell, Pittsburgh, PA). Samples were plated using 100 µL of 10^{-1} to 10^{-3} dilutions per plate. For leaf washings amended with Xcc, Cu^R A44 was added to the washing to yield 10^4 cfu mL^{-1} before shaking. Plates were incubated for 96 h at 28^{o}C before assessment of bacterial growth.

The efficiency of the prospective semi-selective medium for recovering Cu^R or Sm^R Xcc was validated by plating pure cultures of Cu^R or Sm^R Xcc and washings from citrus leaves infected with these Xcc strains on the prospective medium. Assays using pure cultures of Xcc strains Cu^R A44, Sm^R 306S or Cu^S/Sm^S 306 s were conducted as previously described. For assays using leaf washings, young grapefruit leaves were infiltrated with the above strains of Xcc at low concentrations (ca. 7 x 10^{-2} cfu mL^{-1}) to obtain individual lesions that simulate natural infections and lesion development on leaves. Because Cu^R and Sm^R strains of Xcc have not been found in Florida, naturally infected leaves could not be used to test for recovery of resistant strains to such chemicals on the selective media.

Leaf washings were prepared as described earlier. Samples were plated by spreading 100 µL of 10^{-1} to 10^{-3} dilutions per plate. Bacterial counts were performed 96 h after plating by assessing total number of Xcc colonies per plate and the presence of

non-target colonies. Asymptomatic leaves from citrus groves were combined with the inoculated leaves at the ratio of 50% to increase diversity and concentration of microorganisms in the sample to be plated on the prospective selective media, which was expected not only to suppress other microorganisms from citrus phyllosphere, but also to allow typical growth of Xcc in the presence of selective components.

Monitoring for the Presence of Resistant Populations of *Xanthomonas citri* subsp. *citri* and Epiphytic Bacteria on Young Citrus Trees Treated with Copper or Streptomycin

Trial description

The study was conducted in a commercial citrus grove with endemic citrus canker located in Fort Pierce, in Southeast Florida (latitude 27^{o} 29' N, longitude 80^{o} 25' W and altitude 11 m) using 'Ray Ruby' grapefruit (*Citrus paradisi* Macfad) trees grafted on Swingle citrumelo planted at spacing of 3.7 × 7.6 m (approximately 360 trees per hectare) . The trial started in 2008, when trees were 3 years old, and was conducted for three years.

The experiment was arranged in a completely randomized block design with five replicates per treatment and five trees per replicate (25 trees per treatment). Trees were treated with either copper, streptomycin or kept untreated (untreated control – UTC). Experimental design and plot locations remained unchanged during the three seasons.

Trees were sprayed with copper hydroxide (Kocide® 3000 - 30% metallic copper) at 6.32 g per tree or streptomycin sulfate (Firewall® - 22.3% a.i.) at 4.69 g per tree on foliage every 21 days from March to October of each season. Approximately 3.0–3.8 L per tree of spray, depending on the tree size, was applied with a with a handgun sprayer (Chemical Containers Inc., Lake Wales, FL) at 1380 kPa of air pressure. UTC trees were sprayed every 21 days with water only.

Sampling and evaluations

Epiphytic bacteria

Citrus leaves were sampled monthly during the spray period of the first and second seasons (2008 and 2009, respectively) to assay for Cu^R or Sm^R epiphytic bacteria residing in the phyllosphere. Four mature, canker-asymptomatic leaves were collected from different quadrants of each tree in the plot. In both seasons the first and last samplings were conducted before the first and after the last sprays, respectively. Leaves from the same plot were bulked and washed under the same conditions previously described elsewhere in this chapter. Washings from the two treatments and UTC were diluted in tap water and 100 µL of the 10^{-1}, 10^{-2} and 10^{-3} dilutions were plated onto plain MGY agar and MGY agar amended either with 200 mg L^{-1} of copper or 100 mg L^{-1} of streptomycin sulfate. Cycloheximide was added to all plates at 50 mg L^{-1} to suppress fungal growth. After 96 h of incubation at 28^oC, total colonies were counted on all three media. Cu^R and Sm^R epiphytic bacterial populations were expressed as the percentage of the number of colonies per gram of leaf obtained on MGY amended with copper or streptomycin, respectively, in comparison with the colony count present on plain MGY agar, which was also used to determine total bacterial population in the phyllosphere.

Xanthomonas citri subsp. *citri*

The development of Cu^R or Sm^R Xcc in sprayed trees was assessed monthly from March to October in the first two seasons as for the epiphytic bacteria and in May, June and July in the third season. For each assessment, 1 to 4 canker-symptomatic citrus leaves per tree from the most recent mature flush were sampled from plots treated with copper or streptomycin. Leaves from the same plot were bulked and washed in MGY

broth as described previously. Washings were diluted in tap water and plated onto the semi-selective medium MGY-KCH at 10^{-2}, 10^{-4} and 10^{-6} dilutions for determining total Xcc population, and onto MGY-KCH amended with 75 mg L^{-1} of copper or 100 mg L^{-1} of streptomycin at 10^{-1}, 10^{-2} and 10^{-3} dilutions to assess for the presence of Cu^R or Sm^R strains of Xcc. Plates were incubated for 96 h at 28^{o}C before assessing for the presence of Xcc. Xcc-like colonies were subcultured overnight in NA at 28^{o}C and infiltrated in the mesophyll of grapefruit leaves at 10^8 cfu mL^{-1} to check for pathogenicity. Suspects were also streaked onto MGY agar amended with 200 mg L^{-1} of copper or 100 mg L^{-1} of streptomycin to confirm resistance to these chemicals. For Cu^R suspects, strains were pre-grown overnight on NA + 20 mg L^{-1} of copper at 28^{o}C for induction of resistance before plating on MGY + 200 mg L^{-1} of copper (Basim et al., 2005).

Disease assessment

The efficacy of copper and streptomycin for control of citrus canker was assessed in August and October of the first two seasons by determining the incidence of canker-symptomatic leaves and premature leaf drop (Behlau et al., 2010). Six mature branches from all quadrants of each of the three innermost trees in the plots had the total number of leaves, leaves with canker and leaf scars quantified. Whenever possible, the most recent mature flushes on the branches (approximately 3–6 weeks old) were evaluated. Defoliation was assessed in October of each season only and was estimated as the number of leaf scars present on the branch compared with the total of leaves initially presented on the branch (leaves present + scars observed). Both incidence of diseased leaves and defoliation were transformed to percentage data.

Data analysis

The percentage of Cu^R or Sm^R epiphytic bacteria and the logarithm of total bacterial population recovered from trees treated with copper or streptomycin were plotted over time for the two seasons assessed and compared at each evaluation by the standard error of the mean. Treatments were contrasted at the end of the season using the area under the progress curve (AUPC) of the percentage of Cu^R or Sm^R epiphytic bacteria (Campbell and Madden, 1990). AUPC of citrus canker incidence and defoliation were compared among treatments by analyzing the variance with ANOVA and comparing the averages with Tukey's test using SAS—Statistical Analysis System (SAS Institute, Cary, NC).

Screening Bacteria from the Citrus Phyllosphere for Copper Resistance Genes

Isolation of citrus phyllosphere bacteria

Cu^R epiphytic bacterial strains were isolated from the citrus phyllosphere by washing mature canker-asymptomatic citrus leaves as previously described and plating on MGY agar amended with 200 mg L^{-1} of copper. MGY agar was supplemented with cycloheximide at 50 mg L^{-1} to suppress fugal growth. Citrus leaf samples used in this study were collected from citrus commercial groves regularly treated with copper located in Fort Pierce, FL and Immokalee, FL in May and September 2007, respectively. After spreading 100 µL of 10^{-1} to 10^{-3} dilutions of the leaf washings on MGY agar, plates were incubated for 96 h at 28^{o}C. For isolation, single colonies obtained on MGY + copper were subcultured on NA for culture purification and stored in sterile tap water in 1.5 mL microfuge tubes at RT for further use. Strains were identified through fatty acid analysis as previously described (Graham et al., 1999). Fatty acid methyl esters were separated and profiles were identified using the Microbial Identification (MIDI) System

(Microbial ID, Inc. Newark, DE) in the Department of Plant Pathology, University of Florida, Gainesville. A similarity index was used to express the similarity of the test strain and strains in the library of fatty acid profiles stored in the MIDI Library Generation Software. Based on the fatty acid identification the gram negative strains were selected and screened for Cu^R genes. A Cu^R epiphytic strain of *Xanthomonas* sp. (INA69) stored in glycerol 20% at -80^oC isolated from the phyllosphere of a Valencia sweet orange tree in Leesburg, Lake County, Florida in 1984, was included in this study.

PCR analysis

Fifty-three selected gram negative bacterial strains from the citrus phyllosphere (Table 3-5) were screened for the presence of Cu^R genes using PCR (Polymerase Chain Reaction) analysis. The oligonucleotide primer sequences used in this experiment were designed based on the Cu^R genes *copL*, *copA* and *copB* identified in Xcc A44 (Table 3-3) as described in Chapter 4.

Primers were synthesized by Sigma-Aldrich (Sigma-Aldrich Co., St. Louis, MO). Amplification of target genes from all bacteria was performed using a DNA thermal cycler (MJ Research PTC 100, Cambridge, MA) and the Taq polymerase kit (Promega, Madison, WI). For extraction of template DNA, strains were individually grown overnight on NA, suspended in sterile deionized water (DI) in pools of 5 strains per suspension, boiled for 15 min, cooled on ice for 5 min, centrifuged at 15,000 rpm for 5 min and kept on ice to use the supernatant in the PCR reaction mixture. Strains from pools that yielded positive results were further analyzed individually. Each PCR reaction mixture, prepared in 25 µL total volume, consisted of 10.3 µL of sterile water, 5 µL of 5 × PCR buffer, 1.5 µL of 25 mM $MgCl_2$, 4 µL deoxyribonucleoside triphosphates (0.8 mM each dATP, dTTP, dGTP, and dCTP), 0.5 µL of each primer (stock concentration, 25 pmol

μL^{-1}), 3 µL of template, and 0.2 µL (5 U/µl) of Taq DNA polymerase. PCR reactions were initially incubated at 95°C for 5 min. This was followed by 30 PCR cycles which were run under the following conditions: denaturation at 95°C for 30 s, primer annealing at 60^{o}C for all set of primers for 30 s, and DNA extension at 72°C for 45 s in each cycle. After the last cycle, PCR tubes were incubated for 10 min at 72°C and then at 4°C. Cu^{R} Xcc A44 alone and a 5-strain pool spiked with A44 were used as positive controls. PCR reaction mixtures were analyzed by 2% agarose gel electrophoresis (Bio-Rad Laboratories, Hercules, CA) with Tris-acetate-EDTA (TAE) buffer system. A 50-bp DNA ladder (Promega, Madison, WI) was used as the standard molecular size marker for PCR product sizing. Reaction products were visualized by staining the gel with ethidium bromide (0.5 µg mL^{-1}) for 20 min and then photographed using a UV transilluminator and Quantity One software (Bio-Rad Universal Hood II, Hercules, CA).

Horizontal Transfer of Copper and Streptomycin Resistance Genes

Bacterial strains

Horizontal gene transfer of copper and streptomycin resistance genes was investigated within different species of *Xanthomonas* and from citrus epiphytic bacteria to *Xanthomona*s. Rifamycin resistant (Rif^{R}) and spectinomycin resistant ($Spec^{R}$) double mutants of Cu^{S} Sm^{S} strains of Xcc, Xac, *Xanthomonas vesicatoria* (Xv) and *Xanthomonas perforans* (Xp) were used as recipients (Tables 3-5 and 3-6). Cu^{R} strains of Xcc, Xac, Xv, Xp and *X.* sp. INA69, an epiphytic *Xanthomonas* strain isolated from the citrus phyllosphere, as well as eleven Gram-negative non-*Xanthomonas* strains resistant to copper and/or streptomycin isolated from the citrus phyllosphere were used as donors (Tables 3-5 and 3-6).

These non-*Xanthomonas* epiphytic strains were selected from the collection of strains screened for copper resistance genes as previously described in this chapter. The selected epiphytic strains initially identified through fatty acid analysis had the identity rechecked using 16S rRNA gene sequencing method (Han, 2006). Sequences obtained for the unknown organisms were compared to 16S data in Genbank using Basic Local Alignment Search Tool for Nucleotides (BLASTN), National Center for Biotechnology Information (NCBI), USA (Altschul et al, 1990).

Conjugation assays were also conducted using washings of citrus leaves collected in May, July and September 2009 from grapefruit trees treated with copper or streptomycin every 21 days from March to November 2008 and from March until the assessment month in 2009. These washings were used for monitoring the development of Cu^R and Sm^R bacterial populations on grapefruit trees sprayed with these chemicals as detailed previously in this chapter. Washings from each of the five plots per treatment were mated separately with the two recipient strains tested in the three months evaluated (Table 3-6), resulting in15 matings per treatment per recipient strain. All other matings were tested three times (Table 3-6).

Conjugation *in vitro*

Bacterial strains were mated in liquid and on solid media. For conjugation on solid medium, which was used in matings involving pure cultures of *Xanthomona* strains *s* with other xanthomonads and *Xanthomonas* with epiphytic bacteria (Table 3-6), a loop of 24 h bacterial culture of donor and recipient strains pre-grown on NA was spot-mixed and grown on NA agar amended with 20 mg L^{-1} of copper at 28^{o}C for 24 h. Bacterial cells were then suspended in sterile tap water before plating.

Conjugation in liquid medium was used only for matings between *Xanthomonas* and citrus epiphytic bacteria (Table 3-6). Epiphytic bacteria were used as pure culture strains and as bacterial suspensions of citrus leaf washings (Table 3-6), which were primarily used in this study for monitoring Cu^R and Sm^R bacterial population on citrus leaves sprayed with these chemicals as detailed previously in this chapter. For matings in liquid media involving pure culture epiphytic bacteria, 2 mL of MGY broth amended with 1 mg L^{-1} of copper were inoculated with a loop of 24 h NA bacterial culture of donor (epiphytic bacteria) and recipient (*Xanthomonas*) strains (Table 3-6) and incubated for 24 h at 28^{o}C under shaking at 200 rpm using a KS10 orbital shaker (BEA-Enprotech Corp., Hyde Park, MA). For conjugation in liquid medium using bacterial suspensions from citrus leaves, 5 mL of washing was spiked separately with Xcc 306 and Xp 91-118. Before adding to the mating broth, these recipient strains were grown overnight on NA, resuspended in sterile tap water and added to the washing to yield 10^4 cfu mL^{-1}, a concentration similar to the epiphytic bacteria in the washing suspension. Tubes were incubated for 24 h at 28^{o}C under constant shaking using a KS10 orbital shaker (BEA-Enprotech Corp., Hyde Park, MA). For each mating, either in liquid or on solid media, 50 and 300 µL of the mating suspension were plated on NA amended with rifamycin (80 mg L^{-1}), spectinomycin (100 mg L^{-1}) and copper (200 mg L^{-1}) or streptomycin (100 mg L^{-1}) for transconjugant selection. To determine the population of the donor strain, 100 µL of the suspensions were also plated at 10^{-4} and 10^{-6} dilutions on NA amended with rifamycin and spectinomycin at the above concentrations. The conjugation frequency was determined as the ratio between the number of transconjugants obtained for a

specific mating and the total population of the recipient strain recovered per mL of mating suspension.

Conjugation *in planta*

Conjugation *in planta* was tested for transfer of copper resistance genes using Xcc and Xac strains only (Table 3-6). Bacterial cells were previously grown overnight on NA, suspended in sterile tap water and infiltrated with a hypodermic needle and syringe into young grapefruit leaves. Recipient and donor strains were infiltrated separately at concentrations of 5 x 10^8 and 10^9 cfu mL^{-1}, respectively. Bacterial cell suspensions were adjusted in a spectrophotometer (Spectronic 20, Baush & Lomb, Inc.) to an OD of 0.3 and 0.6 at 600 nm, corresponding to approximately 5 x 10^8 and 10^9 cfu mL^{-1}, respectively. After infiltration of bacterial suspensions, plants were incubated at 28^{o}C for 72 h in a growth room with a diurnal light cycle of 12 h. Following, four leaf tissue discs of 0.5 cm^2 were cut from each infiltrated leaf and macerated in 2 mL of MGY both amended with 1 mg $mL^{-1}$of copper. From each mating, 200 and 500 µL of bacterial suspension were immediately plated onto NA plates amended with rifamycin (80 mg L^{-1}), spectinomycin (100 mg L^{-1}) and copper (200 mg L^{-1}).

Isolation of plasmid DNA

Transfer of plasmid harboring copper resistance genes from Cu^R to Cu^S strains was substantiated through plasmid profiling. Bacterial strains were grown overnight in 2 mL nutrient broth (NB) at 28^{o}C under agitation at 200 rpm using a KS10 orbital shaker (BEA-Enprotech Corp., Hyde Park, MA). Bacterial cell suspensions were then standardized to an OD of 0.3 A at 600 nm using a spectrophotometer (Spectronic 20, Baush & Lomb, Inc.). Plasmid DNA was extracted following the method of Kado and Liu (1981) with modifications (Minsavage et al., 1990). Detection of plasmids was

performed by electrophoresis as described previously (Minsavage et al., 1990). After extraction, 28 µL of samples were run in a 0.5% agarose gel, stained with ethidium bromide (0.5 µg mL^{-1}) for 30 min and photographed using a UV transilluminator and Quantity One software (Bio-Rad Universal Hood II, Hercules, CA). Plasmids of *Pantoea stewartii* SW2 (syn. *Erwinia stewartii*) were used as molecular weight markers (Coplin et al. 1981).

Assessment of Copper Resistance in Citrus Epiphytic Bacteria

Epiphytic bacterial strains isolated from the citrus phyllosphere previously used for conjugation assays and screened for Cu^R resistance genes using PCR, as aforementioned in this chapter, were characterized regarding the ability to grow and/or survive at different concentrations of copper on solid medium and in water. Cu^R and Cu^S strains of Xcc and Xac were included in this study as reference strains. Copper sulfate pentahydrate ($CuSO_4.5H_2O$) was used for the copper resistance assessments and strains were maintained on NA prior to the assays.

For tests on solid medium, strains were grown overnight on NA amended with 20 mg L^{-1} of copper for induction of resistance (Basim et al., 2005), suspended in sterile tap water at approximately 5 x 10^8 cfu mL^{-1} and then spotted in triplicate on MGY agar supplemented with 0, 25, 50, 100, 150, 200, 300, 400, 600, and 800 mg L^{-1} of copper. Plates were incubated for 96 h prior to assessment of bacterial growth. The level of copper resistance was determined by comparing bacterial growth on MGY amended with various concentrations of copper and on MGY alone.

For assessment in water, strains were previously induced on NA + 20 mg L^{-1} of copper as described above and added to a final concentration of 10^3 cfu mL^{-1} into 5 mL of sterile distilled (DI) water amended with 0.01 M of magnesium sulfate ($MgSO_4$) and

copper at 0, 1, 2, 4, and 8 mg L^{-1}. Test tubes were kept at 28^{o}C under agitation at 200 rpm using a KS10 orbital shaker (BEA-Enprotech Corp., Hyde Park, MA). Bacterial suspensions were sampled at 0, 1, 2 , 4, 8, and 24 h after exposing to copper by plating 100 µL onto NA. Plates were incubated at 28^{o}C for 96 h prior to assessment of growth.

Expression of *copLAB* from *Stenotrophomonas maltophilia* in *Xanthomonas*

The gene cluster *copLAB* was PCR amplified from *Stenotrophomonas maltophilia* strain FB03P (Stm FB03P) isolated from the phyllosphere of a grapefruit tree and introduced through triparental mating into Cu^{S} *Xanthomonas* species. Primers CopLABF (5'- GCGTGACTT TGTCCGTGAACTC-3') and CopLABR (5'- CGCACCTCAATGGAA CGCTC-3'), designed based on the sequence of copper resistance determinants from Xcc A44 (Chapter 4), were used to amplify the 3.7 kp gene cluster from FB03P.

Before cloning, the PCR product was purified using the QIAquick PCR purification kit (Qiagen) according to the manufacturer's instructions. Purified PCR product was cloned into pGEM-T Easy vector (Promega, Madison, WI) following manufacturer's instructions and then cut from pGEM using *Eco*RI and introduced into pLAFR3 (Staskawicz et al., 1987) to obtain pStmCu1. Ligations were performed with T4 DNA ligase (Promega, Madison, WI) as described by the manufacturer. Ligation products were transformed into competent cells of *Escherichia coli* DH5α produced by the calcium chloride procedure as described by Sambrook et al. (1989). pStmCu1 was then conjugated from *E. coli* DH5α into Rif^{R} and $Spec^{R}$ copper sensitive (Cu^{S}) strains of Xcc 306, Xac 1390, Xp 91-118, and Xv 82-8 by triparental matings with pRK2013 as the helper plasmid (Figurski and Helinski, 1979). Matings were carried out by mixing mid-exponential-phase cells of the recipient strain ME24 with cosmid donor and with pRK2073 on NYG agar (Turner et al., 1984) at the ratio of 2:1:1 (vol/vol/vol) of recipient,

donor and helper strains, respectively. After 24 h of incubation at 28°C, the mating mixtures were resuspended in 2 mL of mannitol-glutamate yeast extract (MGY) broth amended with 1 mg L^{-1} of copper for induction of copper resistance. Aliquots of 50 µL were spread onto nutrient agar (NA) plates containing kanamycin and tetracycline for selection of transconjugants. Plates were incubated for 96 h at 28^{o}C. Transconjugants were then grown overnight on NA amended with 20 mg L^{-1} of copper for induction of resistance to copper (Basim et al., 2005) and suspended in sterile tap water at approximately 10^{8} cfu mL^{-1}. Suspensions were spotted (10 µL) on NA amended with 0, 25, 50, 75, 100, 150, 200, 300, and 400 mg L^{-1} of copper sulfate pentahydrate and evaluated for resistance to copper after 96 h of incubation at 28^{o}C.

Introduction of Stm FB03P *copLAB* on pStmCu1plasmid into Cu^{S} strains of *Xanthomonas* was confirmed individually for each gene by PCR using primers designed for *copL*, *copA*, and *copB* from Xcc A44 (Table 3-3), as mentioned earlier in this chapter. Finally, the 3.7 kb *copLAB* gene cluster from Stm FB03P in pGEM and PCR products from each gene obtained from the transconjugants were sequenced and compared. DNA sequencing was performed by the DNA Sequencing Core Laboratory of the Interdisciplinary Center for Biotechnology Research (ICBR), University of Florida, Gainesville. For the 3.7 kb *copLAB* in pGEM, sequencing was initiated using the standard flanking vector F20 and R24 primers. Custom primers designed based on the sequences obtained with F20 and R24 primers were used to complete the sequencing. Sequencing of individual PCR products of *copL*, *copA*, and *copB* was performed with primers used for PCR analysis (Table 3-3). Sequences were then aligned using Clustal W (Thompson et al., 1994).

Results

Development of a Semi-Selective Medium for the Isolation of Copper and Streptomycin Resistant Strains of *Xanthomonas citri* subsp. *citri* from Plant Material

Amendment of MGY with kasugamycin, cephalexin and chlorothalonil at previously reported concentrations (Graham and Gottwald, 1990) did not affect recovery and growth of Cu^R and Cu^S Xcc. Recovery of Cu^R Xcc on MGY-KCB amended with 50 mg L^{-1} of copper was comparable to MGY-KCB and MGY alone (Figure 3-1A). Although Cu^S Xcc was fully recovered on NGA-KCB, recovery of Cu^R Xcc was reduced in the presence of KCB and completely suppressed when copper was added to NGA-KCB (Figure 3-1A), leading to the selection of MGY as the basal medium. Color of Xcc colonies on MGY is slightly different than observed on NGA. On MGY Xcc colonies are light yellow or pale yellow, whereas on NGA colonies have a brighter and vivid yellow appearance (Figure 3-1A). No other differences in the colony characteristics were observed. Suppression of fungal growth on MGY-KC agar was more satisfactory when chlorothalonil (Bravo 720) was replaced by cycloheximide (H) (Figure 3-1B).

Additional antibiotics were tested but were determined to be unsuitable for incorporating into MGY-KCH medium for several reasons. 5–Fluorouracil reduced growth and recovery of Xcc when added to MGY-KCH (Figure 3-1C). Boric acid and tobramycin reduced growth of Cu^R Xcc in the presence of copper (Figure 3-1D) and, even when these two antibiotics were used at lower concentrations and Xcc was fully recovered on MGY-KCH, no improvement in suppression of contaminants and selectivity for Xcc was observed (Figure 3-1E). The use of higher concentrations of cephalexin (Figure 3-1F) and kasugamycin in MGY than used by Graham and Gottwald

(1990) either impaired or prevented growth of Cu^R Xcc. Satisfactory suppression of fungal contaminants was obtained with cycloheximide at 50 mg mL^{-1}.

The resistance level of Cu^R Xcc to copper on MGY was reduced in the presence of KCH. As observed elsewhere in this chapter, A44 can grow on MGY amended with up to 400 mg L^{-1} of copper. However, this level was reduced to 100 mg L^{-1} when the medium was amended with KCH (Figure 3-1G). Satisfactory suppression of Cu^S Xcc and confluent growth of Cu^R Xcc was observed when MGY-KCH was amended with 75 and 100 mg L^{-1} of copper (Figure 3-1G). Likewise, 100 mg L^{-1} of streptomycin allowed and prevented efficient growth of Sm^R and Sm^S Xcc, respectively. Recovery of different Cu^R or Sm^R strains of Xcc or other species of *Xanthomonas* on MGY-KCH amended with copper or streptomycin was comparable to MGY alone (Figure 3-1H).

MGY agar amended with 16 mg L^{-1} of kasugamycin (K), 16 mg L^{-1} of cephalexin (C), and 50 mg L^{-1} of cycloheximide (H) provided the most favorable results and the final medium was designated MGY-KCH. It allowed selective growth of Cu^R and Sm^R strains of Xcc in the presence of copper at 75 mg L^{-1} or streptomycin at 100 mg L^{-1}, respectively (Figure 3-2; Table 3-2) and suppressed other microorganisms naturally present in the citrus phyllosphere (Figure 3-2).

Monitoring for the Presence of Resistant Populations of *Xanthomonas citri* subsp. *citri* and Epiphytic Bacteria on Young Citrus Trees Treated with Copper or Streptomycin

No significant difference in total epiphytic bacterial population on trees sprayed with copper or streptomycin was observed over time in comparison to the UTC (Figure 3-5). From March to October in the two years assessed, total bacterial populations recovered from citrus leaves varied from 1.4×10^4 to 1.9×10^6 cfu per gram of plant material (Figure 3-5). Copper and streptomycin sprays increased the ratios of epiphytic

bacterial populations with resistance to these chemicals in the two seasons studied (Figure 3-3). The frequency of Cu^R bacteria on trees sprayed with copper was significantly higher than UTC and streptomycin treated trees for most of the assessments from the third month of sprays (May) to the end of the season (October) for both years studied (Figure 3-3 A and B). Likewise, Sm^R bacterial population on streptomycin treated trees increased significantly from the fourth month (June) to the last month sprayed (October) (Figure 3-3 C and D). These trends were significant for the AUPC of resistant epiphytic bacteria (Figure 3-4). The AUPC's of Cu^R and Sm^R epiphytic bacteria for trees treated with copper and streptomycin, respectively, were statistically greater than the AUPC's for the UTC (Figure 3-3). Overall, the frequency of Sm^R epiphytic bacteria on treated and untreated leaves was proportionally lower than the Cu^R bacterial population (Figure 3-3).

No Cu^R or Sm^R Xcc was recovered from citrus trees treated with these chemicals for three consecutive seasons. The total population of Xcc recovered from canker-symptomatic leaves ranged from 2×10^5 to 3×10^7 cfu per gram of leaf. Citrus canker incidence on leaves and defoliation were significantly lower on copper treated trees (Figure 3-6). In October 2008 the differences were evident (Figure 3-7) and the percentage of leaves with canker for copper treated trees and UTC was 21.7 and 70.8, respectively. In October 2009, the incidence followed the same trend observed in the previous year and reached 36.4% and 82.4% on treated and untreated trees, respectively (Figure 3-6 A and B). As a consequence of the high incidence of citrus canker, defoliation observed for UTC in October of 2008 (Figure 3-8) and 2009 was more than four-fold higher than for copper treated trees (Figure 3-6C). While leaf drop

for UTC reached 35% and 29% in 2008 and 2009, respectively, defoliation did not exceed 8% for trees treated with copper during the same periods (Figure 3-6C). Disease incidence and defoliation were intermediate for streptomycin treated trees and did not differ statistically from the UTC (Figure 3-6).

Screening Bacteria from the Citrus Phyllosphere for Copper Resistance Genes

Of 53 epiphytic strains isolated from citrus trees tested using PCR, only strains INA69 and FB03P harbored copper resistance genes homologous to those found in Cu^R Xcc. INA69 is an epiphytic *Xanthomonas* and FB03P is *Stenotrophomonas maltophilia*. The *Xanthomonas* strain was isolated from the phyllosphere of a Valencia sweet orange tree in Leesburg, Lake County, Florida in 1984 and the *S. maltophilia* strain is from a grapefruit grove in Fort Pierce, Saint Lucie County, Florida obtained in 2007. INA69 and FB03P tested positive for the three genes involved in copper resistance that are described in Chapter 4. The sizes of PCR products obtained for these two strains using primers designed for resistance genes *copL*, *copA,* and *copB* were comparable to the sizes observed for Cu^R Xcc A44 (Figure 3-9). No DNA amplification was observed for any of the *cop* genes for the other epiphytic strains tested.

Horizontal Transfer of Copper and Streptomycin Resistance Genes

Conjugation assays demonstrated that copper resistance genes are likely harbored on large (~300 kb) conjugative plasmids (Figure 3-10) which can be exchanged within different species of *Xanthomonas* (Table 3-7). Cu^R genes were shown to move from Xcc to Xcc, Xac and Xp, from Xac to Xac, Xcc and Xp, from Xp to Xp, Xcc, and Xac, and from Xv to Xv and Xcc (Table 3-7). Conjugation frequency of copper resistance genes ranged from 10^{-7} to 10^{-5} transconjugants per recipient cell (Table 3-7). On the contrary, conjugation of Cu^R genes did not occur from Xcc, Xac, and Xp to Xv

and from Xv to Xac and Xp (Table 3-7). Likewise, conjugation assays *in planta*, or involving epiphytic bacteria, either as isolated strains or from leaf washings, were negative for recovery of Cu^R or Sm^R transconjugant strains on the selective medium.

Assessment of Copper Resistance in Citrus Epiphytic Bacteria

The selected Cu^R epiphytic bacterial strains isolated from citrus groves treated with copper behaved differently when exposed to several concentrations of copper on solid medium and in water (Figures 3-11 and 3-13). On MGY agar, Cu^S and Cu^R control strains of *Xanthomonas* grew up to 75 and 400 mg L^{-1} of copper, respectively (Figure 3-11). Among the epiphytic strains, the highest and lowest resistance levels were observed for strains FB38P of *Luteibacter yeojuensis* and FB35P of *Sphingomonas* sp., respectively (Figures 3-11). While the former strain grew on MGY amended with up to 800 mg L^{-1} of copper, the latter could not grow at concentrations higher than 200 mg L^{-1} (Figure 3-11). Nine of the 12 epiphytic strains tested were able to grow at 400 mg L^{-1} or higher concentrations of copper on solid medium (Figure 3-11).

When the same strains were exposed to copper in water, *Sphingomonas* sp. FB49P, which was one of the least resistant on MGY agar, showed the lowest levels of resistance and could not be recovered after 2 h of exposure at any of the copper concentrations tested (Figure 3-13). By contrast, *Methylobacterium* sp. strains FB10P and FB61P and *Sphingomonas melonis* strain FB70P showed the highest resistance. For these strains, the number of viable cells increased at lower concentrations of copper and remained unchanged or declined slightly after being exposed to 8 mg L^{-1} of copper for 24 h (Figure 3-13). Cu^S negative control strains Xcc 306 and Xac 1390 were not recovered after 4 h of exposure to any of the concentrations of copper tested (Figure 3-12). By contrast, survival of control strains resistant to copper, Xcc A44 and Xac 1381 in

water amended with copper was inversely proportional to the concentration of copper in solution (Figure 3-12). Viable cells of Xcc A44 and Xac 1381 were recovered on NA after exposure to 1, 2 and 4 mg L^{-1} of copper for 24 h. However, these bacteria could not be recovered after 8 h at 8 mg L^{-1} of copper (Figure 3-12). Resistance levels observed on solid medium and in water for these two strains was comparable to strains INA69 and FB03P (Figures 3-11 to 3-12), which were shown to harbor homologues of copper resistance genes found in *Xanthomonas* A44 and 1381 (Figure 3-9).

Expression of *copLAB* from *Stenotrophomonas maltophilia* in *Xanthomonas*

Introduction of *copLAB* gene cluster from Stm FB03P through triparental mating conferred copper resistance to Cu^S strains of Xcc, Xac, Xp, and Xv. Transconjugants were able to grow on MGY agar supplemented with 200 mg L^{-1} of Cu after 96 h of incubation. As a reference, the sensitive strains used as recipients can grow on MGY amended with up to 50 - 75 mg L^{-1} of Cu.

Sequence analysis of the 3.7 kb *copLAB* cloned gene cluster (GenBank accession number HM636054) confirmed the presence of ORFs *copL*, *copA*, and *copB,* which indicates accuracy of PCR amplification and confirms the similarities of *copLAB* from Stm FB03P and Xcc A44, as detailed in Chapter 4. Moreover, PCR analyses confirmed the presence of each of the three genes in all recipient strains. Nucleotide sequences of PCR products of pStmCu1 in Xcc 306 harboring the copper resistance genes from Stm FB03P were 100% identical with the 3.7 kb fragment originally cloned and used for conjugations.

Discussion

The newly developed semi-selective medium, MGY-KCH, amended with copper or streptomycin was satisfactory for recovery of Cu^R and Sm^R strains of Xcc from plant

material previously inoculated with known resistant strains. As is typical of most semi-selective media, the efficiency of MGY-KCH for suppressing phyllosphere microorganisms was somewhat variable, ranging from complete inhibition of contaminants to high selectivity of Xcc. Several antibiotics could not be added to the medium as selective agents for recovery of Xcc because the presence of copper increased sensitivity of Xcc to certain compounds. For this reason, the level of copper added to MGY-KCH had to be lowered from 200 mg L^{-1}, a standard concentration used to assess copper resistance of Xcc *in vitro* (Basim et al., 2005) to 75 mg L^{-1}. Such a concentration was high enough to suppress copper sensitive strains of Xcc and select for the Cu^R ones. The use of MGY-KCH amended with copper or streptomycin to screen resistant strains of Xcc alleviates the need for isolating the pathogen from plant material prior to testing for resistance to the compounds. Thus the semi-selective medium was a very useful tool for efficiently screening for the presence of Cu^R and Sm^R strains of Xcc in citrus groves.

The development of strains resistant to copper has been reported for many bacterial pathogens affecting crops (Adaskaveg and Hine, 1985; Andersen et al., 1991; Bender and Cooksey, 1986; Cazorla et al., 2002; Cooksey et al., 1990; Marco and Stall, 1983; Martin et al., 2004; Ritchie and Dittapongpitch, 1991 Scheck and Pscheit, 1998; Stall et al., 1986; Sundin et al., 1989). However, in the present study, no Cu^R strain of Xcc was isolated from citrus trees sprayed with a copper bactericide every 21 days for 3 consecutive seasons. Due to the nature of the genetics of copper resistance in bacteria, which is conferred by several genes normally organized in operons (Cooksey, 1990; Mellano and Cooksey, 1988a; Voloudakis et al., 2005), a natural spontaneous mutation

conferring copper resistance is unlikely to occur within bacterial populations. Conjugation of plasmid or transposable elements carrying such resistance genes is likely to be the main means for enabling the development of copper resistance in bacterial populations (Bender and Cooksey, 1986; Bender et al., 1990; Stall et al., 1986). This is a process difficult to track in nature and is highly dependent on many local environmental factors such as cell density (Levin et al., 1979; Normander et al., 1998), growth phase (Muela et al., 1994), temperature (Khalil and Gealt, 1987) as well as pH, cations, salinity, dissolved oxygen, and nutrient availability (Khalil and Gealt, 1987; Roszak and Colwell, 1987). Long history of exposure to copper bactericides is a common factor identified in previous reports of the development of Cu^R in Xcc (Canteros, 1996) and other bacterial plant pathogens (Adaskaveg and Hine, 1985; Andersen et al., 1991). The relatively short period that Xcc population was exposed to copper during this study (3 seasons) and before that, due to the recent adoption of copper sprays for control of citrus canker in Florida after the eradication program was halted in 2005, may have accounted for the absence of Cu^R strains of Xcc in symptomatic trees repeatedly treated with copper in this study.

As observed for copper, no Sm^R strains were isolated after citrus trees had undergone 3 seasons of 21-day-interval sprays of streptomycin in the present study. The efficacy of streptomycin sprays for control of citrus canker has been tested as a complementary measure to copper bactericides routinely used in citrus producing areas with endemic occurrence of the disease (Graham et al., 2008). Streptomycin is widely used in apple and pear groves for control of bacterial blight caused by *Erwinia amylovora*. However, the development of resistant strains to this antibiotic due to

intensive use has been reported from many areas in the United States (Coyier and Covey 1975; Miller and Schroth, 1972; Schroth et al. 1979; Shaffer and Goodman 1985) and Canada (Sholberg et al., 2001) and has hampered disease control. Streptomycin has not been used in commercial citrus groves and the development of Xcc strains resistant to this antibiotic in the field has not been reported. Resistance to streptomycin develops either by horizontal transfer of resistance genes or by mutation (Gale et al., 1981; Springer et al., 2001). The latter is the more common mechanism of streptomycin resistance acquisition and occurs through a single base-pair mutation of the streptomycin binding site (Springer et al., 2001). Evidences for these two processes have been demonstrated previously in plant pathogenic bacteria (Burr et al., 1988; Schroth et al., 1979). Occurrence of Sm^R mutant strains is rare in nature due to reduced fitness (Schroth et al., 1979). However, continued use of this antibiotic in the field for control of plant disease after the emergence of resistant genotypes allows the mutant bacteria to compensate for lack of fitness associated with the newly acquired resistance genes (Schroth et al., 1979). Thus, although a number of factors can contribute to the development of bacterial populations resistant to streptomycin, clearly the selection pressure posed by regular sprays of the antibiotic and random mutations are the most important. Thus, although Sm^R strains of Xcc were not found in the present study after 3 seasons of sprays, previous studies indicate that development of resistance in the Xcc population could occur any time. With continued use of streptomycin, resistance development is inevitable due to incessant mutation and selection in bacterial populations (Moller et al. 1981). What remains to be addressed is how likely Sm^R strains

will develop in Xcc populations if only a few streptomycin sprays are intercalated or mixed with copper applications for control of citrus canker.

Although no Cu^R or Sm^R strains of Xcc were found, the frequent sprays of copper and streptomycin increased the population of epiphytic bacteria resistant to these chemicals residing in the citrus phyllosphere. The increased frequency is likely to reflect changes in community structure, adaptation of the initial community as well as selection of resistant populations initially present. Previous studies reported a correspondence between exposure to bactericides and the frequency of resistant strains to these chemicals in the environment (Berg et al., 2005; Kunito et al. 1999; Smit et al. 1997). In the present study, total bacterial population in the phyllosphere did not differ between copper or streptomycin treated trees and untreated control. Therefore Cu^R and Sm^R bacterial communities may have taken over the sensitive ones, which were suppressed by the frequent bactericide sprays. Considering that cell density plays an important role in conjugation frequency (Levin et al., 1979; Normander et al., 1998), the concern for build-up Cu^R and Sm^R bacterial communities in the phyllosphere is that it increases the likelihood for exchange of resistance genes. Consequently, there is greater risk for the development of resistant strains of Xcc to these chemicals.

As reported previously for other *Xanthomonas* (Bender et al., 1990; Cooksey et al., 1990; Stall et al., 1986), the Cu^R genes in Xcc are located on large conjugative plasmids. We showed that Cu^R genes can be transferred between different plant pathogenic species of *Xanthomonas* and that homologous of these resistance genes present in epiphytic bacteria residing on the citrus phyllosphere can confer copper resistance to sensitive strains of *Xanthomonas*. Therefore, despite that the movement of

copper or streptomycin resistance genes from epiphytic strains to *Xanthomonas* could not be demonstrated in the present study, it is possible that in nature phyllosphere microorganisms represent a risk for the development of resistance in the Xcc population. In *Erwinia amylovora*, causal agent of fireblight on pear and apple, mobilizable streptomycin resistance genes have been previously identified in common epiphytic bacteria found in orchards (Beining et al., 1996; Burr et al., 1988; Huang and Burr, 1999; Norelli et al., 1991; Sobiczewski et al., 1991). The common plasmid-borne streptomycin resistance genes *strA-strB* genes have been well characterized in populations of epiphytic bacteria that coexist in close proximity to *E. amylovora* (Huang and Burr, 1999; Sobiczewski et al., 1991). According to Sundin (2002), *strA-strB* genes can be carried within an integron, a transposon, or on broad-host-range plasmids. This genetic exchange has facilitated the world-wide dissemination of this determinant for streptomycin resistance among at least 21 bacterial genera (Sundin, 2002). Cooksey et al. (1990) reported the presence of Cu^R saprophytic *Pseudomonas putida* strains that harbor plasmid borne resistance genes homologous to those in *P. syringae* pv. *tomato* from a commercial tomato seed lot. In addition to the contribution of seedborne saprophytic bacteria to the spread of resistant bacterial populations between fields and between different geographical areas, the results reported here illustrate that copper resistance genes can potentially be shared between pathogenic *Xanthomonas* sp. and non-pathogenic epiphytic bacteria in the citrus phyllosphere.

Table 3-1. Steps used for development of a semi-selective medium for the recovery of copper or streptomycin resistant strains of *Xanthomonas citri* subsp. *citri* from plant material.

Step	Purpose [a]	Medium Tested [b, c]	Selective components (mg L^{-1}) [c]	Sample plated [a]
Basic medium	Verify the ability of Xcc to grow on NGA and MGY agar amended with KCB and Cu	NGA NGA + KCB NGA + KCB + Cu MGY MGY + KCB MGY + KCB + Cu	K (16) C (16) B (12) Cu (50)	Pure culture of Xcc Cu^R A44 and Cu^S 306
Chlorothalonil versus cycloheximide	Asses the efficacy of fungicides chlorothalonil and cycloheximide to suppress fungal growth and allow confluent growth and selection of Xcc in the presence of Cu	MGY MGY + KC + B MGY + KC + B + Cu MGY + KC + H MGY + KC + H + Cu	K (16) C (16) B (12) H (40) Cu (50)	Pure culture of Xcc Cu^R A44 and Cu^S 306, citrus leaf washing amended or not with Cu^R A44.
Additional antibiotics	Evaluate the efficiency of other antibiotics to improve suppression of contaminants and allow confluent growth and selection of Xcc	MGY MGY + KCH MGY + KCH + FBo MGY + KCH + FBoT	K (16) C (16) H (40) F (6,12) Bo (150, 300) T (0.20, 0.40)	Pure culture of Xcc Cu^R A44 and Cu^S 306, citrus leaf washing amended or not with Cu^R A44.
Concentration of KCH	Verify the effect of different concentrations of KCeCy on the suppression of contaminants and growth of Xcc in the presence of Cu	MGY MGY + KCH + Cu	K (16, 32), C (16, 32, 60) H (50, 100)	Pure culture of Xcc Cu^R A44 and Cu^S 306, citrus leaf washing amended or not with A44.
Cu concentration	Assess the optimal concentration of Cu that suppress Cu^S strains and allow Cu^R strains to grow in the presence of antibiotics/fungicides.	MGY + KCH MGY + KCH + Cu	K (16) C (16) H (50) Cu (25, 50, 75, 100, 150, 200)	Pure culture of Xcc Cu^R A44 and Cu^S 306

Table 3-1. Continued

Sm concentration	Assess the optimal concentration of Sm that suppress Sm^S strains and allow Sm^R strains to grow in the presence of antibiotics/fungicides.	MGY + KCH MGY + KCH + Sm	K (16) C (16) H (50) Sm (25, 50, 75, 100)	Pure culture of Xcc Sm^R 306S and Sm^S 306
Different strains and species	Evaluate growth of different strains and different species of Cu^R or Sm^R *Xanthomona*s on the newly developed semi-selective media, MGY-KCH	MGY + KCH MGY + KCH + Cu MGY + KCH + Sm	K (16) C (16) H (50) Cu (75) Sm (100)	Pure culture of Cu^R Xcc A26 and A16, Xv 81-23, Xac 1381 and Sm^R Xac 1620[c]

[a] Cu^R/Sm^R and Cu^S/Sm^S indicate copper (Cu) or streptomycin (Sm) resistant and sensitive strains, respectively; Xcc, *Xanthomonas citri* subsp. *citri*; Xv, *Xanthomonas vesicatoria*; Xcc, *Xanthomonas alfalfae* subsp. *citrumelonis*;
[b] MGY, mannitol-glutamate yeast extract agar; NGA, nutrient glucose agar;
[c] K, kasugamycin; C, cephalexin; H, cycloheximide; F, 5–fluorouracil; T, tobramaycin; Bo, boric acid; B, chlorothalonil (Bravo 720); Cu, copper sulfate pentahydrate ($CuSO_4.5H_2O$); Sm, streptomycin sulfate.

Table 3-2. Recovery of *Xanthomonas citri* subsp. *citri* (Xcc) on MGY-KCH amended or not with copper or streptomycin.

Medium[a]	Xcc Strain		
	306 (Cu^S/Sm^S)[b]	A44 (Cu^R/Sm^S)	306S (Cu^S/Sm^R)
MGY	100.0	100.0	100.0
MGY-KCH	92.8[c]	103.6	92.3
MGY-KCH Cu	0.0	96.4	0.0
MGY-KCH Sm	0.0	0.0	94.0

[a] MGY, mannitol-glutamate yeast extract agar; K, kasugamycin (16 mg L^{-1}); C, cephalexin (16 mg L^{-1}); H, cycloheximide (50 mg L^{-1}); Cu, copper sulfate pentahydrate - $CuSO_4.5H_2O$ (75 mg L^{-1}); Sm, streptomycin sulfate (100 mg L^{-1}).
[b] Cu^R/Sm^R and Cu^S/Sm^S indicate copper (Cu) or streptomycin (Sm) resistant and sensitive strains, respectively;
[c] Percentage relative to recovery on plain MGY agar.

Table 3-3. Oligonucleotide primer sets used for screening citrus phyllosphere bacteria for the presence of copper resistance genes *copL*, *copA* and *copB*.

Gene	Primer name	Sequence (5' to 3')	Length (bp[a])	GC[b] (%)	Tm[c] (^{o}C)	Product size (bp)
copL	copLF	CCGTGTCAAGCCTCCTCACTTCTAC	25	56	63	~360
	copLR	CAGCGGCATGACATCCAGGCC	21	67	63	
copA	copAF	CCTCCATGGCACGGACACTTCCATC	25	60	65	~870
	copAR	CCAGACATATCCATCGACCCATGATCCA	28	50	63	
copB	copBF	CTCAGGATCACTCTGCACATCAG	23	52	60	~535
	copBR	GCACGTAGCTCTTAATCGAGTTGTC	25	48	60	

[a] bp, base pair;[b] guanine (G) and cytosine (C) content; [c] calculated melting temperature.

Table 3-4. Copper resistant bacterial strains isolated from the citrus phyllosphere and screened for copper resistance genes.

Organism [a]	No. of strains tested
Novosphingobium capsulatum	17
Sphingobium yanoikuyae	11
Novosphingobium subterraneum	8
Methylobacterium rhodesianum	4
Brevundimonas vesicularis	3
Xanthobacter flavus	3
Lysobacter enzymogenes	1
Methylobacterium zatmanii	1
Pantoea ananatis	1
Sphingomonas adhaesiva	1
Sphingomonas sanguinis	1
Stenotrophomonas maltophilia	1
Xanthomonas sp.	1

[a] Based on the highest index of similarity obtained in the fatty acid analysis.

Table 3-5. Bacterial strains used in conjugation assays.

Organism/Strain	Function in mating	Relevant characteristic [a]	Geographical origin
Xanthomonas citri subsp. *citri*			
A44	Donor	Cu^R	Argentina
306	Recipient	Cu^S; $Spec^R$; Rif^R	Brazil
Xanthomonas alfalfae subsp. citrumelonis			
1381	Donor	Cu^R	Florida
1390	Recipient	Cu^S; $Spec^R$; Rif^R	Florida
Xanthomonas perforans			
1-7	Donor	Cu^R	Florida
91-118	Recipient	Cu^S; $Spec^R$; Rif^R	Florida
Xanthomonas euvesicatoria			
81-23	Donor	Cu^R	Florida
82-8	Recipient	Cu^S; $Spec^R$; Rif^R	Florida
Bacterial strains from the citrus phyllosphere			
Xanthomonas sp. INA69	Donor	Cu^R	Florida
Spingomonas sp. FB02P	Donor	Cu^R; Sm^R	Florida
Stenotrophomonas maltophilia FB03P	Donor	Cu^R	Florida
Methylobacterium sp. FB10P	Donor	Cu^R	Florida
Naxibacter sp. FB18P	Donor	Cu^R	Florida
Spingomonas sp. FB35P	Donor	Cu^R; Sm^R	Florida
Luteibacter yeojuensis FB38P	Donor	Cu^R	Florida
Spingomonas sp. FB49P	Donor	Cu^R; Sm^R	Florida
Methylobacterium sp. FB61P	Donor	Cu^R	Florida
Spingomonas melonis FB70P	Donor	Cu^R; Sm^R	Florida
Spingomonas sp. FB74P	Donor	Cu^R; Sm^R	Florida

[a] Cu^S, copper sensitive; Cu^R copper resistant; Sm^R, streptomycin resistant; $Spec^R$, spectinomycin resistant; Rif^R, rifamycin resistant.

Table 3-6. List of conjugation assays tested.

Recipient strain	Xcc 306	Xac 1390	Xv 82-8	Xp 91-118	Conjugation tested [a]
Donor strain					
Plant pathogenic *Xanthomonas* [b]					
Xcc A44	VS, P [c]	VS, P	VS	VS	Cu
Xac 1381	VS, P	VS, P	VS	VS	Cu
Xv 81-23	VS	VS	VS	VS	Cu
Xp 1-7	VS	VS	VS	VS	Cu
Epiphytic bacteria strains					
INA69	VS, VL	VS, VL	VS, VL	VS, VL	Cu
FB2P	VS, VL	VS, VL	VS, VL	VS, VL	Cu, Sm
FB3P	VS, VL	VS, VL	VS, VL	VS, VL	Cu
FB10P	VS, VL	VS, VL	VS, VL	VS, VL	Cu
FB18P	VS, VL	VS, VL	VS, VL	VS, VL	Cu
FB35P	VS, VL	VS, VL	VS, VL	VS, VL	Cu, Sm
FB38P	VS, VL	VS, VL	VS, VL	VS, VL	Cu
FB49P	VS, VL	VS, VL	VS, VL	VS, VL	Cu, Sm
FB61P	VS, VL	VS, VL	VS, VL	VS, VL	Cu
FB70P	VS, VL	VS, VL	VS, VL	VS, VL	Cu, Sm
FB74P	VS, VL	VS, VL	VS, VL	VS, VL	Cu, Sm
Epiphytic bacteria from citrus leaf washings (LW) [d]					
LW from Cu treated trees	VL	nt [e]	nt	VL	Cu
LW from Sm treated trees	VL	nt	nt	VL	Sm

[a] Conjugation assays were separately tested for the transference of either copper (Cu) or streptomycin (Sm) resistance genes.
[b] Xcc, *Xanthomonas citri* subsp. *citri*; Xac, *Xanthomonas alfalfae* subsp. *citrumelonis*; Xv, *Xanthomonas vesicatoria*; Xp, *Xanthomonas* perforans.
[c] VS, *in vitro* conjugation on solid medium; VL, *in vitro* conjugation in liquid medium; P, conjugation *in planta*.
[d] Conjugation assays were conducted using washing of citrus leaves collected in May, July and September 2009 from grapefruit trees treated with copper or streptomycin every 21 days from March to November 2008 and from March to the assessment month in 2009. Washings from each of the five plots per treatment were mated separately with the two recipients strains tested in three months evaluated, resulting in15 matings per treatment per recipient strain.
[e] nt, not tested.

Table 3-7. Conjugation frequency of copper resistance genes between different plant pathogenic *Xanthomonas* species.

Donor strain [a]	Recipient strain	Conjugation frequency [b]
Xcc A44	Xcc 306	5×10^{-6} to 1×10^{-5}
	Xac 1390	1×10^{-6} to 2×10^{-6}
	Xp 91-118	1×10^{-7} to 6×10^{-7}
	Xv 82-8	0
Xac 1381	Xcc 306	1×10^{-8} to 1×10^{-7}
	Xac 1390	5×10^{-6} to 2×10^{-5}
	Xp 91-118	2×10^{-8} to 2×10^{-7}
	Xv 82-8	0
Xp 1-7	Xcc 306	3×10^{-6} to 1×10^{-5}
	Xac 1390	8×10^{-6} to 1×10^{-5}
	Xp 91-118	3×10^{-6} to 9×10^{-6}
	Xv 82-8	0
Xv 81-23	Xcc 306	3×10^{-7} to 1×10^{-6}
	Xac 1390	0
	Xp 91-118	0
	Xv 82-8	2×10^{-7} to 1×10^{-6}

[a] Xcc, *Xanthomonas citri* subsp. *citri*; Xv, *Xanthomonas vesicatoria*; Xac, *Xanthomonas alfalfae* subsp. *citrumelonis*; Xp, Xanthomonas perforans.
[b] Number of transconjugant per recipient.

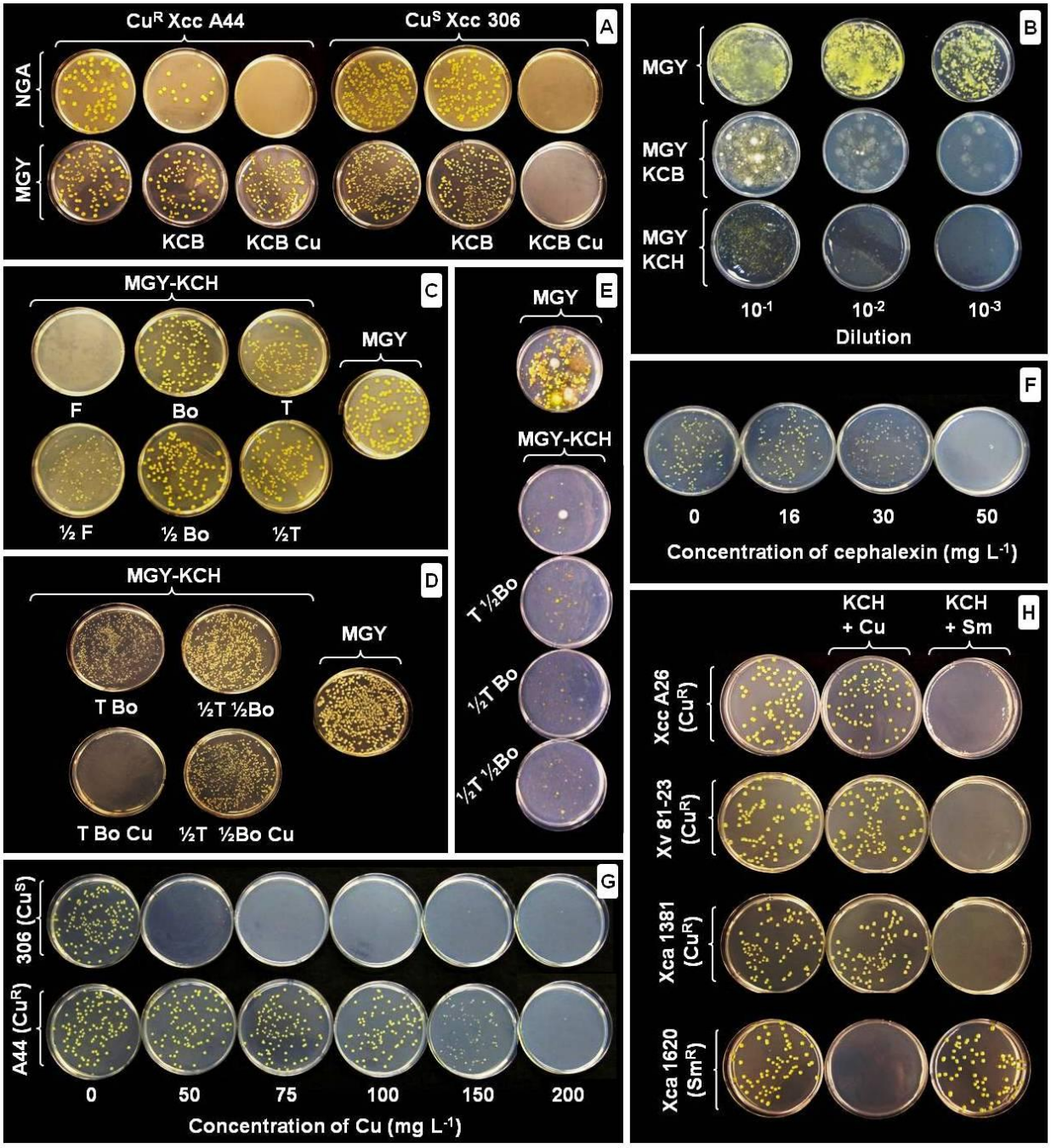

Figure 3-1. Adjustment tests for the establishment of a semi-selective medium for the recovery of copper or streptomycin resistant strains of Xcc from plant material. A) growth of Cu^R A44 and Cu^S 306 Xcc on NGA and MGY agar amended or not with KCB and Cu, B) comparison of chlorothalonil and cycloheximide for suppressing growth of fungal contaminant from leaf washing on MGY amended with KC, C) growth of Cu^R Xcc A44 on MGY and MGY-KCH amended with F, Bo or T, D) growth of Cu^R Xcc A44 on MGY and MGY-KCH amended with Bo and T in the presence and absence of Cu, E) Growth of microorganisms naturally present in the citrus phyllosphere on

Figure 3-1. Continued

MGY and MGY-KCH amended with Bo and T, F) Recovery of Cu^R Xcc A44 on MGY-KH amended with different concentrations of C, G) Recovery of Cu^R and Cu^S Xcc on MGY-KCH amended with different concentrations of Cu, and H) Recovery of a different strain of Xcc and other species of *Xanthomonas* resistant to Cu or Sm on MGY and MGY-KCH amended with Cu or Sm. NGA, nutrient glucose agar; MGY, mannitol-glutamate yeast agar; Cu^R/ and Cu^S/Sm^R indicate copper (Cu) or streptomycin (Sm) resistant and sensitive strains, respectively; Xcc, *Xanthomonas citri* subsp. *citri*; Xv, *Xanthomonas vesicatoria*; Xac, *Xanthomonas alfalfae* subsp. *citrumelonis*; K, kasugamycin (16 mg L^{-1}); C, cephalexin (16 mg L^{-1}); H, cycloheximide (50 mg L^{-1}); F, 5–fluorouracil (6 and 12 mg L^{-1} for ½F and F, respectively); T, tobramaycin (0.2 and 0.4 mg L^{-1} for ½T and T, respectively); Bo, boric acid (150 and 300 mg L^{-1} for ½T and T, respectively); B, chlorothalonil – Bravo 720 (12 mg L^{-1}); Cu, copper sulfate pentahydrate - $CuSO_4.5H_2O$ (50 mg L^{-1} in panel A and D, 75 mg L^{-1} in panel F); Sm, streptomycin sulfate (100 mg L^{-1}).

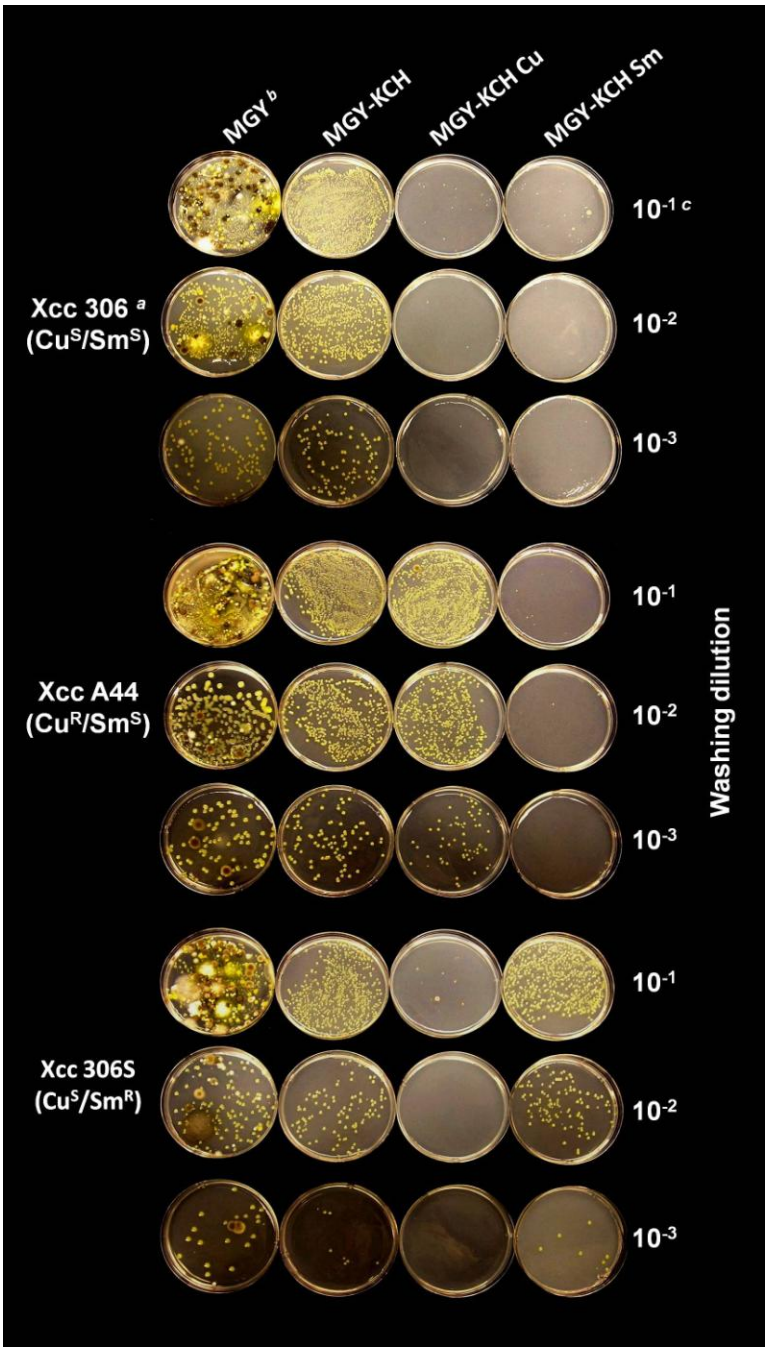

Figure 3-2. Efficiency of MGY-KCH for the selection of copper and streptomycin resistant strains of Xcc (predominant yellow colonies) from washings of inoculated grapefruit leaves. [a] Cu^S/Sm^S and Cu^R/Sm^R indicate copper (Cu) or streptomycin (Sm) sensitive and resistant strains, respectively; Xcc, *Xanthomonas citri* subsp *citri*. [b] MGY, mannitol-glutamate yeast extract agar; K, kasugamycin (16 mg L^{-1}); C, cephalexin (16 mg L-^{1}); H, cycloheximide (50 mg L-^{1}) ; Cu, copper sulfate pentahydrate - $CuSO_4.5H_2O$ (75 mg L^{-1}) ; Sm, streptomycin sulfate (100 mg L^{-1}). [c] 10^{-1}, 10^{-2}, and 10^{-3} indicate 10, 100, and 1000 fold dilutions from leaf washings, respectively.

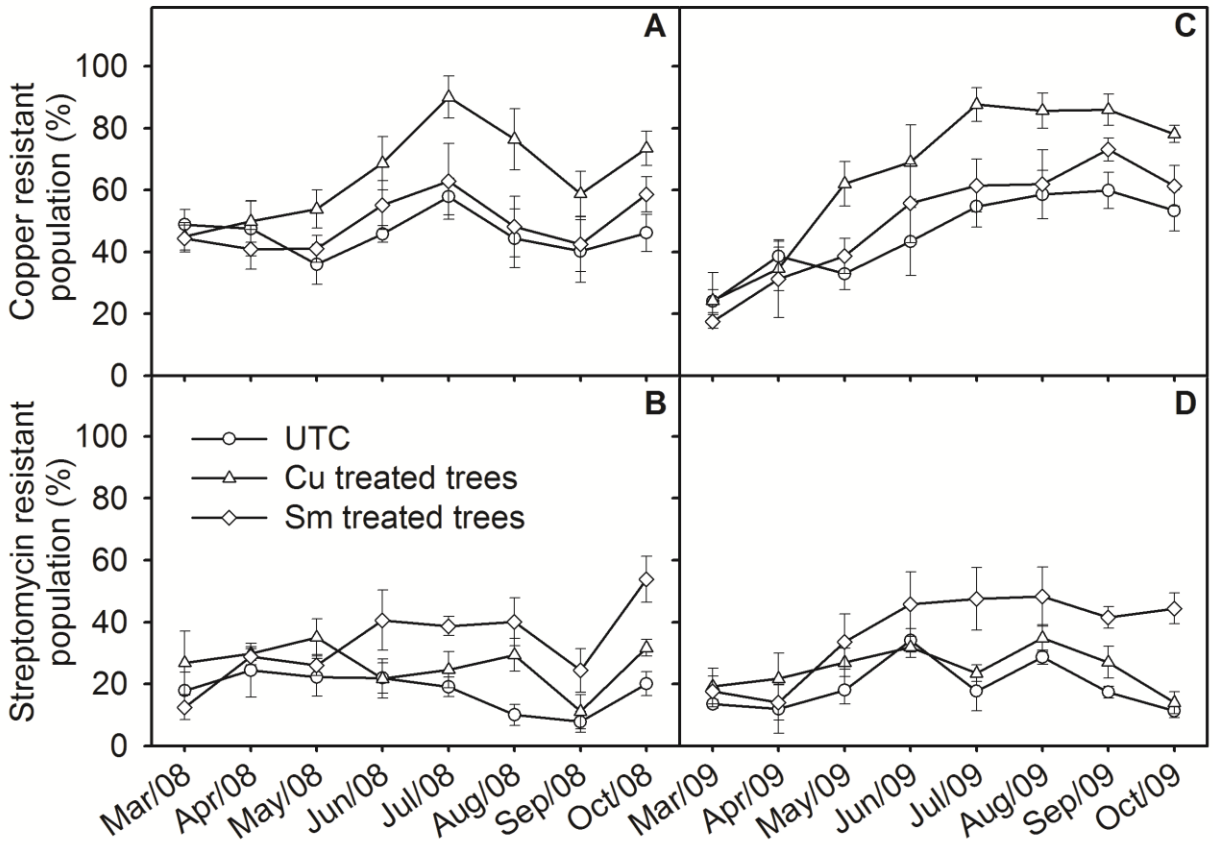

Figure 3-3. Effect of copper (Cu) and streptomycin (Sm) sprays on the epiphytic bacterial population resistant to these chemicals residing on citrus leaves. Frequency of resistant epiphytic bacteria to Cu (A and C) or Sm (B and D) in 2008 and 2009, as percentage of colony forming units recovered on mannitol-glutamate yeast extract agar (MGY) amended with Cu or Sm from trees sprayed with Cu or Sm based bactericides and untreated control (UTC) in comparison to MGY alone. Error bars indicate the standard error of the mean.

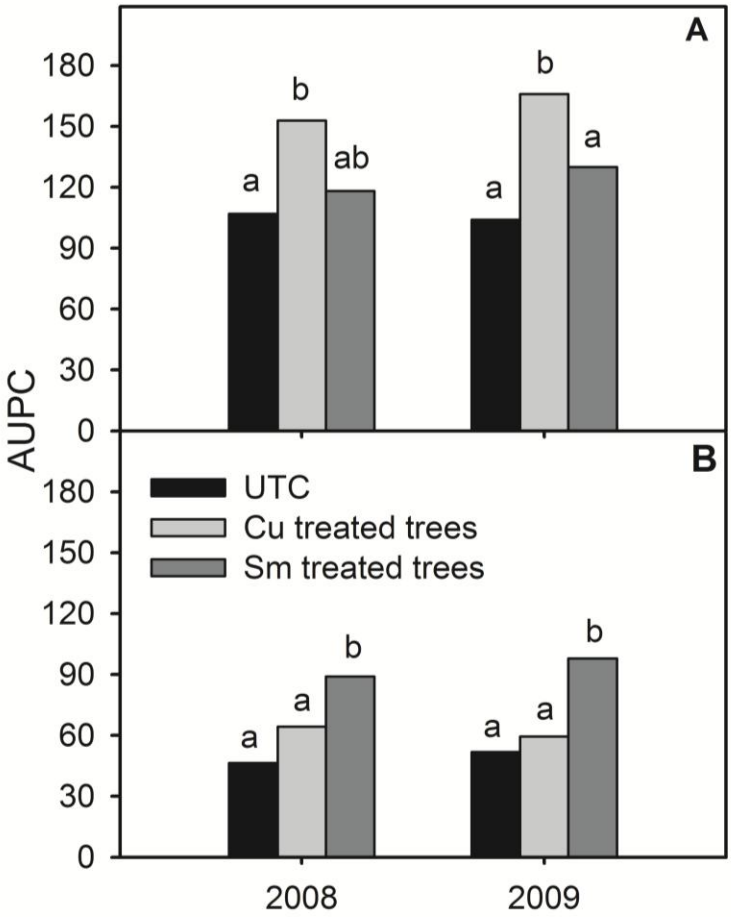

Figure 3-4. Area under the progress curves (AUPC) of percentage of copper (Cu) and streptomycin (Sm) resistant epiphytic bacteria recovered on mannitol-glutamate yeast extract agar (MGY) amended with Cu or Sm from trees sprayed with Cu or Sm based bactericides and untreated control (UTC) in comparison to MGY alone in 2008 and 2009. A) AUPC of epiphytic bacteria resistant to Cu and B) AUPC of epiphytic bacteria resistant to Sm. Means followed by the same letter within the same year are not significantly different by Tukey's test ($P<0.05$).

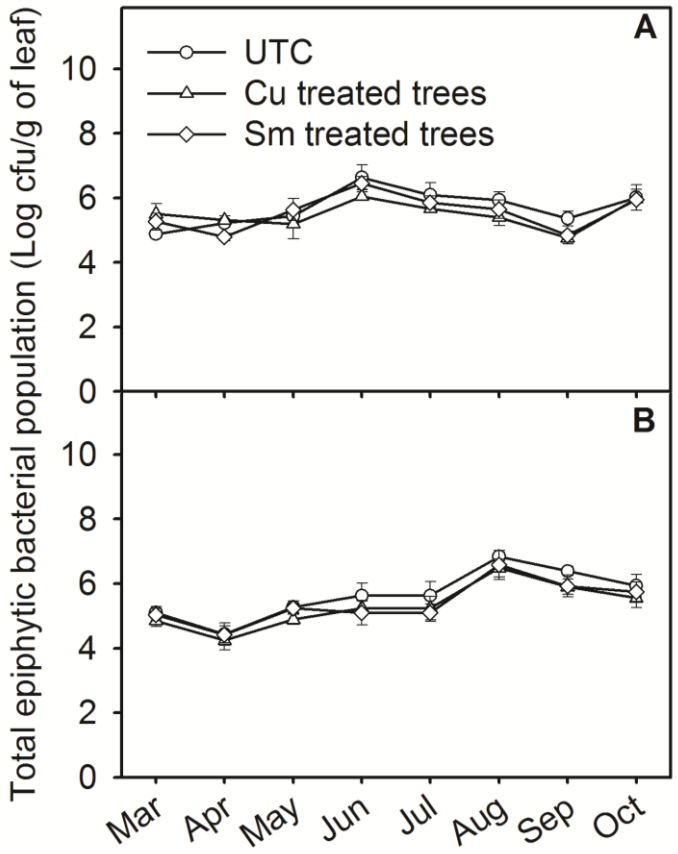

Figure 3-5. Epiphytic bacterial population on citrus trees treated with copper (Cu) and streptomycin (Sm). A and B) total epiphytic bacterial population recovered on mannitol-glutamate yeast extract agar (MGY) from citrus trees treated with Cu or Sm based bactericides and untreated control (UTC) in 2008 and 2009, respectively.

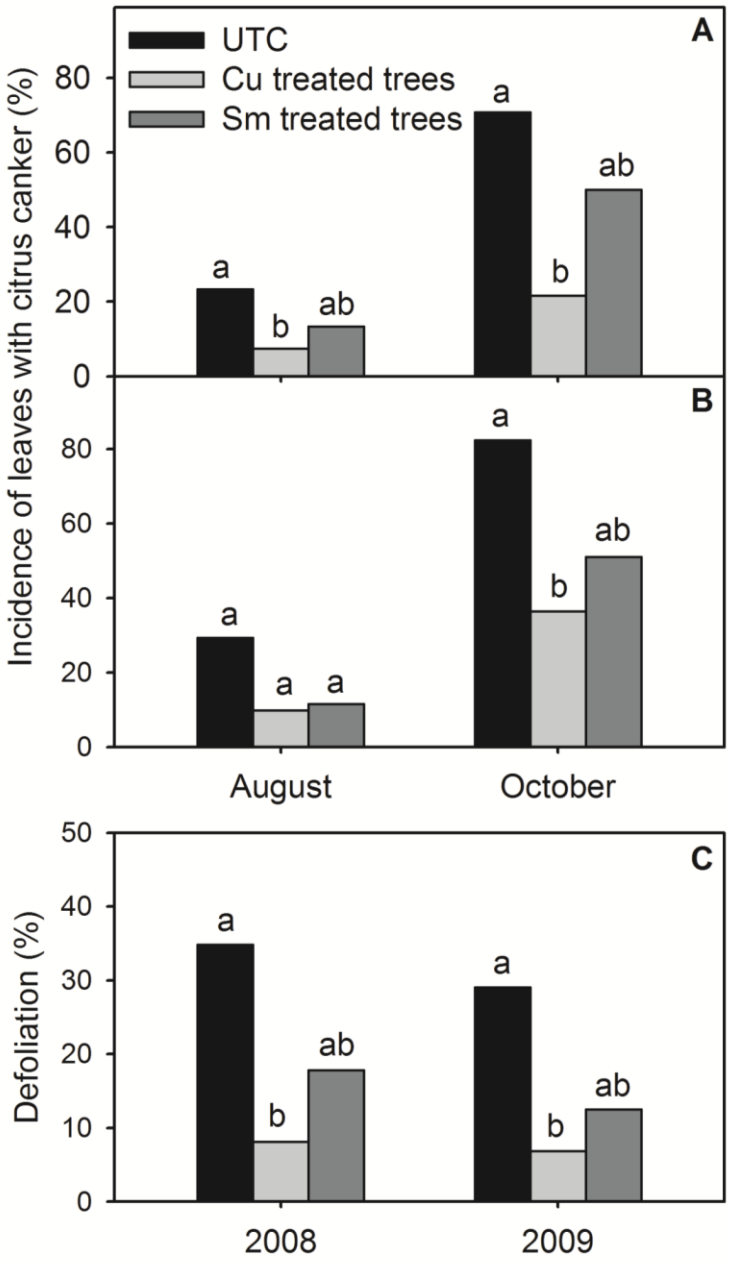

Figure 3-6. Incidence of citrus canker and premature defoliation of citrus trees treated with copper (Cu) or streptomycin (Sm). A and B) incidence of leaves with citrus canker on trees sprayed with Cu or Sm and untreated control (UTC) in 2008 and 2009, respectively and C) premature defoliation of trees treated with Cu or Sm and untreated control in October 2008 and 2009. Means followed by the same letter within the same month in A and B and within the same year in C are not significantly different by Tukey's test ($P<0.05$).

Figure 3-7. Grapefruit trees from the field trial. A) Tree treated with copper (Cu) and B) untreated control. The picture was taken in October 2009, after trees under Cu treatment had been sprayed with this chemical every 21 days from March to October in 2008 and 2009.

Figure 3-8. Premature defoliation of untreated grapefruit trees due to citrus canker in Fort Pierce, FL, 2007.

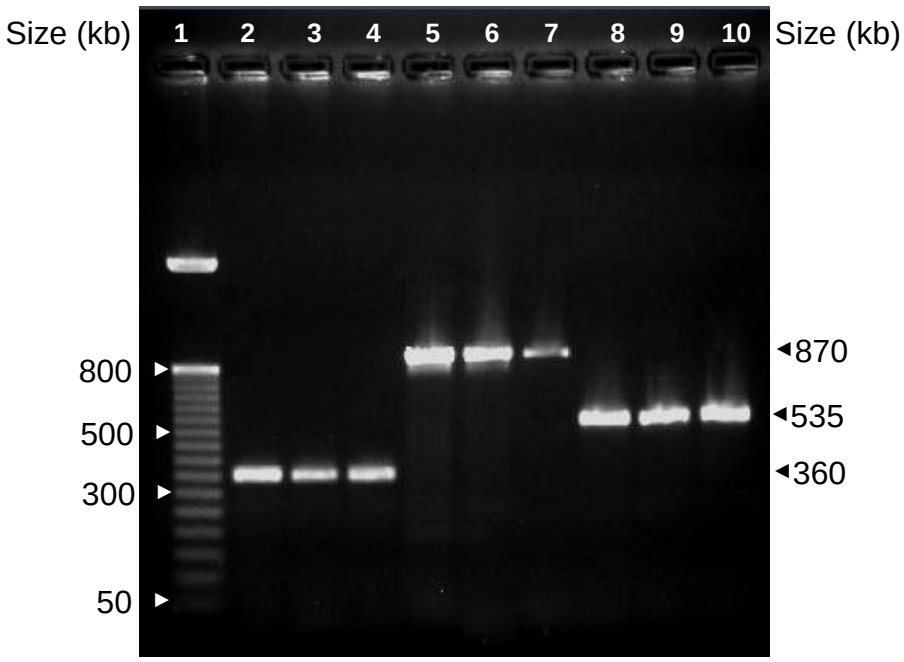

Figure 3-9. Agarose gel electrophoresis of PCR analysis of copper resistance genes *copL*, *copA*, and *copB*. Lanes: (1) marker; (2, 5, and 8) *copL*, *copA*, and *copB* of *Xanthomonas citri* subsp. *citri* A44, respectively; (3, 6, and 9) *copL*, *copA*, and *copB* of epiphytic *Xanthomonas* sp. INA69, respectively; (4, 7, and 10) *copL*, *copA*, and *copB* of *Stenotrophomonas maltophilia* FB03P, respectively. bp, base pair.

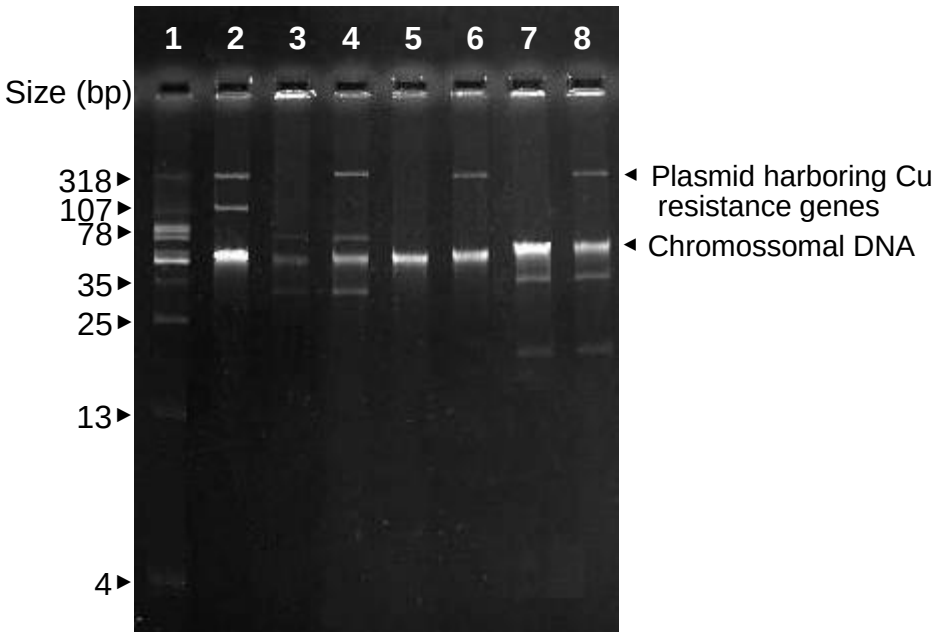

Figure 3-10. Agarose gel electrophoresis of plasmid extractions obtained from copper resistant (Cu^R), copper sensitive (Cu^S) and transconjugant strains of *Xanthomonas*. Lanes:(1) *Pantoea stewartii* used as plasmid size ladder; (2) Cu^R Xcc donor strain A44; (3) Cu^S Xcc recipient strain 306; (4) Cu^R transconjugant of Xcc resulted from the mating between A44 and 306; (5) Cu^S Xac recipient strain 1390; (6) Cu^R transconjugant of Xac resulted from the mating between A44 and 1390; (7) Cu^S Xp recipient strain 91118; (8) Cu^R transconjugant of Xp resulted from the mating between A44 and 91-118. Xcc, *Xanthomonas citri* subsp. *citri*; Xac, *Xanthomonas alfalfae* subsp. *citrumelonis*; Xp, *Xanthomonas perforans.*

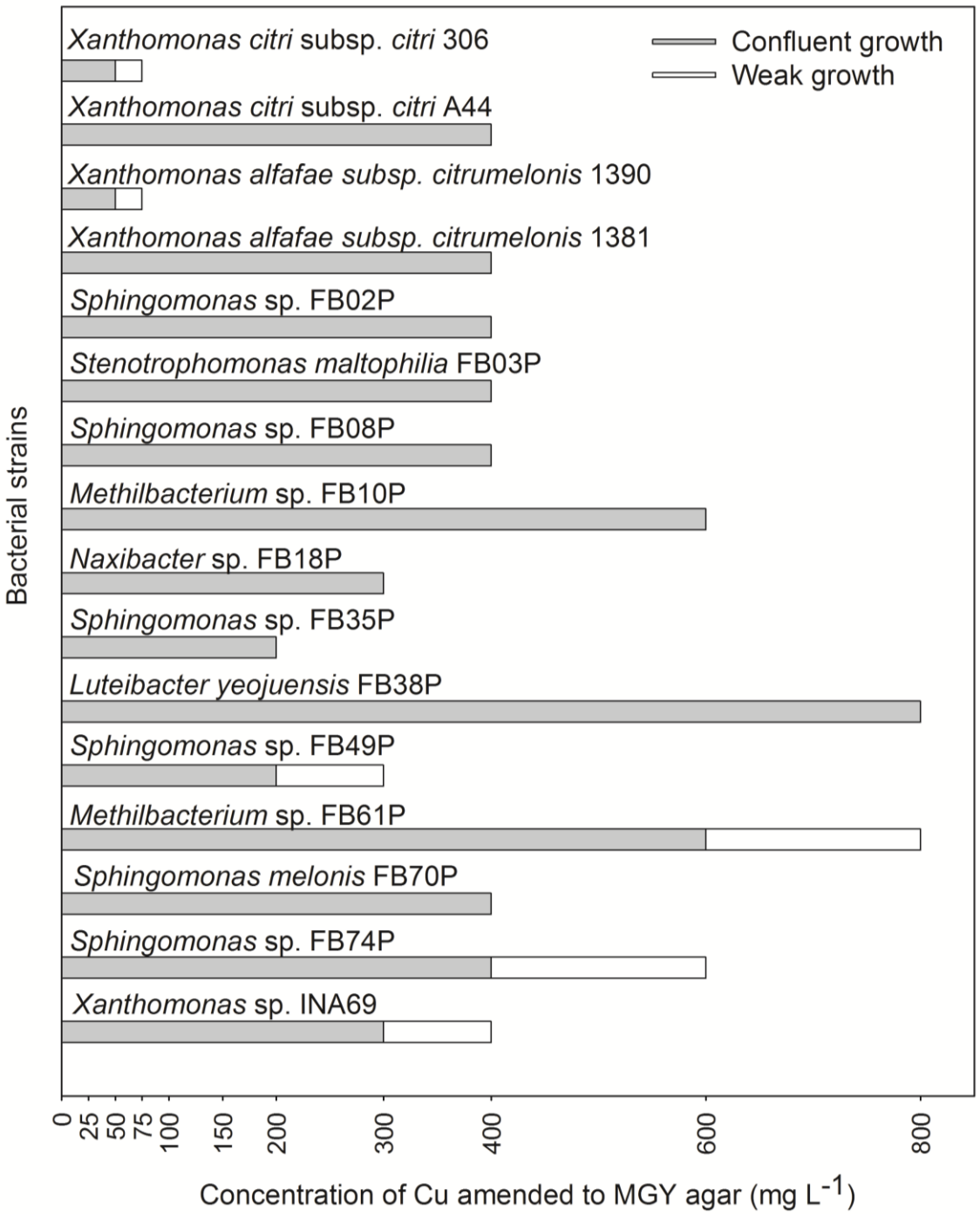

Figure 3-11. Copper resistance levels of selected copper resistant epiphytic bacteria isolated from the citrus phyllosphere and reference strains of *Xanthomonas* sensitive (306 and 1381) and resistant (A44 and 1381) to copper. Cu, copper sulfate pentahydrate ($CuSO_4.5H_2O$); MGY, mannitol glutamate yeast agar.

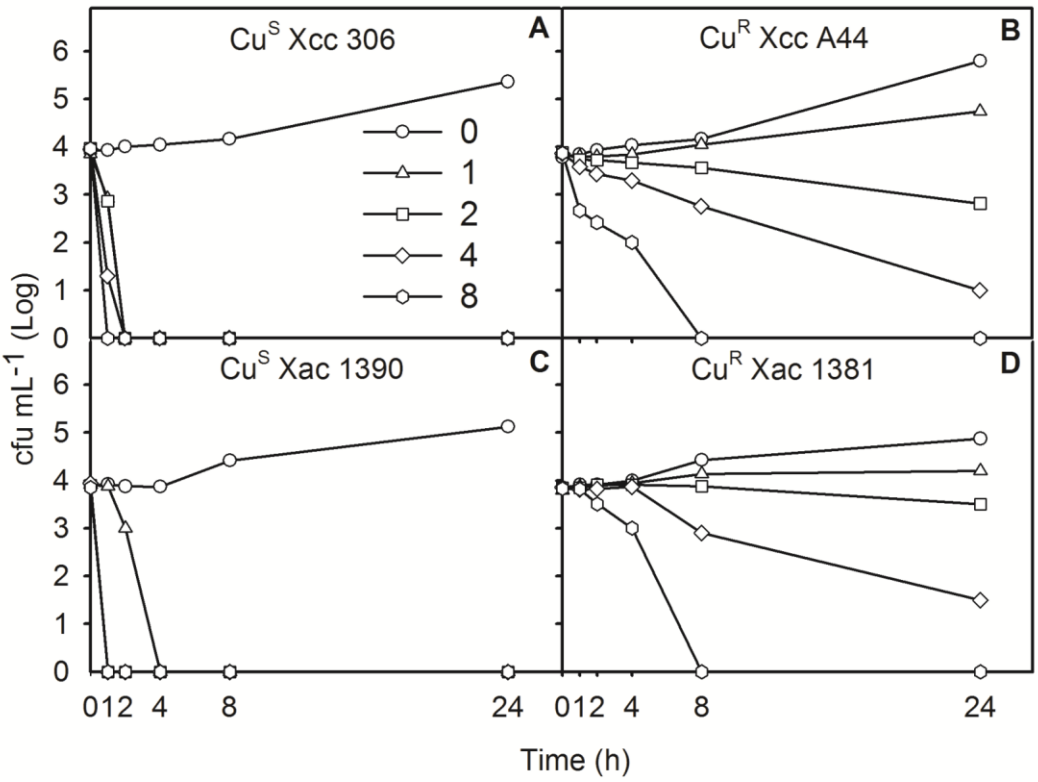

Figure 3-12. Survival of copper sensitive (Cu^S) and copper resistant (Cu^R) strains of plant pathogenic *Xanthomonas* over time in sterile distilled water amended with 0.01 M of magnesium sulfate ($MgSO_4$) and copper sulfate pentahydrate ($CuSO_4.5H_2O$) at 0, 1, 2, 4, and 8 mg L^{-1}. A and B) Cu^S and Cu^R *Xanthomonas citri* subsp. *citri* (Xcc), respectively; C and D) Cu^S and Cu^R *Xanthomonas alfalfae* subsp. *citrumelonis* (Xac), respectively.

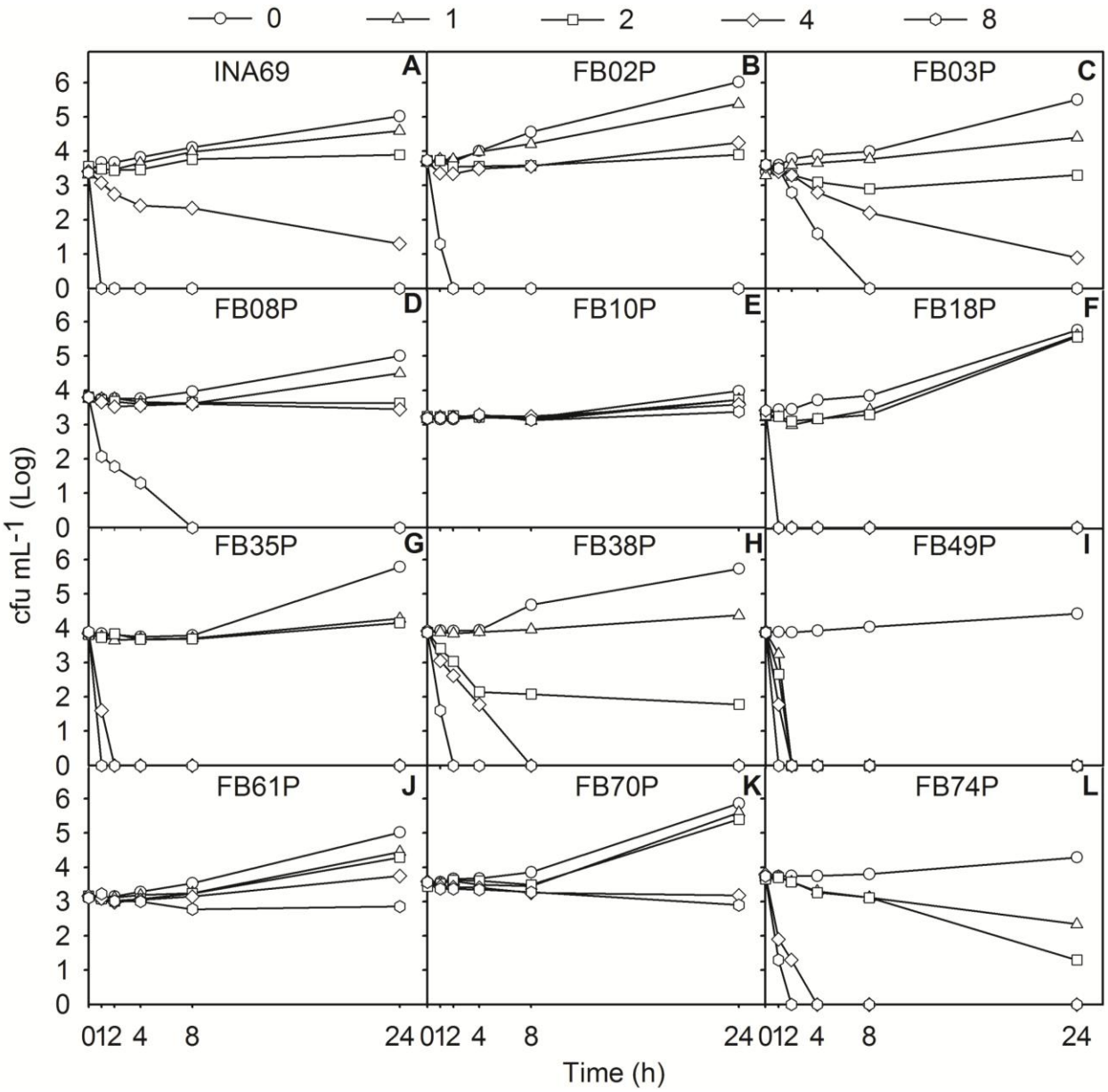

Figure 3-13. Survival of copper resistant epiphytic bacteria isolated from the citrus phyllosphere over time in sterile distilled water amended with 0.01 M of magnesium sulfate ($MgSO_4$) and copper sulfate pentahydrate ($CuSO_4.5H_2O$) at 0 (circle), 1 (triangle), 2 (square), 4 (diamond), and 8 (hexagon) mg L^{-1}. A) *Xanthomonas* sp. INA69, B) *Sphingomonas* sp. FB02P, C) *Stentrophomonas maltophilia* FB03P, D) *Sphingomonas* sp. FB08P, E) *Methylobacterium* FB10P, F) *Naxibacter* sp. FB18P, G) *Sphingomonas* sp. FB35P, H) *Luteibacter yeojuensis* FB38P, I) *Sphingomonas* sp. FB49P, J) *Methylobacterium* FB61P, K) *Sphingomonas melonis* FB70P and L) *Sphingomonas* sp. FB74P.

CHAPTER 4

MOLECULAR CHARACTERIZATION OF COPPER RESISTANCE GENES FROM *Xanthomonas citri* subsp. *citri* AND *Xanthomonas alfalfae* subsp. *citrumelonis*

Introduction

The copious use of copper based bactericides on vegetable and fruit crops for control of bacterial and fungal pathogens has led to the development and prevalence of copper resistant (Cu^R) strains of several species of bacteria affecting plants (Adaskaveg and Hine, 1985; Cooksey et al., 1990; Marco and Stall, 1983; Martin et al., 2004; Ritchie and Dittapongpitch, 1991; Stall et al., 1986; Andersen et al., 1991; Bender and Cooksey, 1986; Cazorla et al., 2002; Scheck and Pscheit, 1998; Sundin et al., 1989). Although, most copper resistance genes characterized from plant pathogenic bacteria have been shown to be plasmid encoded (Carzola et al., 2002; Mellano and Cooksey, 1988; Bender et al., 1990; Bender and Cooksey, 1986; Cooksey, 1987; Cooksey, 1990; Stall et al., 1986; Voloudakis et al., 1993), chromosomal copper resistance genes have also been identified (Basim et al., 2005; Lee et al., 1994; Lim and Cooksey, 1993).

Cellular copper sequestration has been suggested as the copper resistance mechanism in resistant strains of *Pseudomonas syringae* (Cooksey, 1990). In *P. syringae,* the copper resistance operon, *copABCD*, encodes four proteins, CopA, -B, -C, and -D, and is present on plasmid pPT23D (Cha and Cooksey, 1991; Mellano and Cooksey, 1988a). This operon is regulated by a copper inducible promoter that requires the regulatory genes, *copR* and *copS*, located downstream of *copD* (Mills et al., 1993). Mills et al. (1993) suggests that *P. syringae* employs the two component sensory transduction to alter gene expression in response to environmental stimuli and regulate copper resistance gene expression. When grown on copper amended medium, these

strains harboring plasmid pPT23D accumulate copper, indicating that resistance is due to an uptake mechanism (Cooksey, 1994). Studies have shown that *P. syringae* containing the *cop* operon accumulates more copper than strains lacking the operon (Bender and Cooksey, 1987; Cha and Cooksey, 1991; Cooksey and Azad, 1992) and that this operon confers copper resistance to *P. syringae* at least in part by sequestering and accumulating copper in the periplasm with copper binding proteins, which may prevent toxic levels of copper from entering the cytoplasm (Cha and Cooksey, 1991; Cooksey, 1993). According to Rouch et al. (1985), genes that confer copper resistance are regulated and induced only by high levels of copper. Copper inducibility of the pco genes of *Escherichia coli* showed that the lag phase observed upon addition of copper to the growth medium could be reduced by preinduction with copper sulfate (Rouch et al., 1985).

In *E. coli* copper resistance is regulated by different systems, including the multicopper oxidase CueO, which protects periplasmic enzymes from copper mediated damage (Grass and Rensing, 2001), the *cus* determinant, that confers copper and silver resistance (Munson et al., 2000) and the *pcoABCD* operon (Rensing et al., 2000). The latter is known as an efflux mechanism and is responsible for pumping excess copper out of the cytoplasm (Cooksey, 1993). The *pcoABCD* operon shares homology with the *copABCD* operon for *P. syringae* and, as in *P. syringae*, is followed by two regulatory genes, *pcoR* and *pcoS* (Mellano and Cooksey, 1988a).

Copper resistance genes have also been cloned from *Xanthomonas vesicatoria* (Xv) (Cooksey et at.. 1990; Garde and Bender, 1991; Basim et al., 2005), *Xanthomonas arboricola* pv. *juglandis* (Xaj) (Lee et al., 1994) and *Xanthomonas perforans* (Xp)

(Voloudakis et al., 2005). Genetics of the plasmid-borne copper resistance in Xv have similarities to the *cop* operon from *P. syringae* (Voloudakis et al., 1993). Nevertheless, on the chromosome, the organization of the copper resistance genes appears to be uncommon, and occurrence of this type of resistance is rare in Xv (Basim et al., 2005). Copper resistance genes in Xaj are located on the chromosome and have the same general *copABCD* structure as the genes from *P. syringae*, with some differences in DNA sequence and gene size (Lee et al., 1994). In Xp copper resistance genes are plasmid-encoded and expression of these genes was demonstrated to be regulated by *copL*, which is the immediate open reading frame (ORF) upstream of *copAB* (Voloudakis et al., 2005).The *copRS* regulatory genes, which are present in *P. syringae* (Mellano and Cooksey, 1988a), have not been found in *Xanthomonas* (Lee et al., 1994; Voloudakis et al., 2005).

The objective of this study was to characterize the copper resistance determinants in *Xanthomonas citri* subsp. *citri* (Xcc) (syn. *Xanthomonas axonopodis* pv. *citri*) and *Xanthomonas alfalfae* subsp. *citrumelonis* (Xac) (syn. *X. campestris* pv. *citrumelo*, *X. campestris* pv. *citri* strain E), causal agents of citrus canker and citrus bacterial spot, respectively and compare with other copper resistance bacteria.

Material and Methods

Bacterial strains, plasmids, and culture conditions

Bacterial strains and plasmids used in molecular studies and their relevant characteristics and sources are listed in Table 4-1. Cu^R strain A44 from Argentina isolated in 1994 and strain 1381 from Florida isolated in 2000 were used for characterization of the Cu^R genes from Xcc and Xac, respectively. *Xanthomonas* strains were maintained in nutrient agar (NA) at 28^oC, whereas cultures of *Escherichia coli*

were grown in Luria-Bertani (LB) (Maniatis et al., 1982) broth at 37°C. A pLAFR3 cosmid library of Xcc A44 and Xac 1381 was maintained in *E. coli* DH5α on LB medium containing tetracycline. All other strains were stored in sterile tap water at room temperature or in 20% glycerol at -70°C or both. Antibiotics were used to maintain selection for resistance markers at the following final concentrations: ampicillin 100 mg L^{-1}, kanamycin 50 mg L^{-1}, spectinomycin 100 mg L^{-1},rifamycin 80 mg L^{-1}, and tetracycline 12.5 mg L^{-1}. Nutrient broth (NB), and LB broth were used as liquid media to grow *Xanthomonas* and *E. coli*, respectively. Cultures were grown for 24 h at 28°C on a KS10 orbital shaker (BEA-Enprotech Corp., Hyde Park, MA) at 200 rpm. Copper was used as copper sulfate pentahydrate ($CuSO_4.5H_2O$) and added to the liquid and solid medium from a 1 or 50 mg mL^{-1} stock solution, respectively, before autoclaving.

Construction of genomic libraries and isolation of copper resistant clones

A pLAFR3 cosmid (Staskawicz et al., 1987) library of DNA from strains Xcc A44 and Xac 1381 was created as previously described (Maniatis et al., 1982) and maintained in *E. coli* DH5α. Total genomic DNA was extracted using Illustra plasmidPrep Mini Spin Kit (GE Healthcare, Piscataway, N.J.) following manufacturer's instructions. Constructed plasmids were introduced into Kan^R Xp ME24 from *E. coli* DH5α by triparental matings with pRK2013 as the helper plasmid (Figurski and Helinski, 1979). Matings were carried out by mixing mid-exponential-phase cells of the recipient strain ME24 with cosmid donors and with pRK2073 on NYG agar (Turner et al., 1984) at the ratio of 2:1:1 (vol/vol/vol) of recipient, donor and helper strains, respectively. After 24 h of incubation at 28°C, the mating mixtures were resuspended in 2 mL of mannitol-glutamate yeast extract (MGY) broth amended with 1 mg L^{-1} of copper for induction of presumptive copper resistance genes to be screened. Aliquots of 50 µL were spread

onto NA plates containing kanamycin and tetracycline for selection of transconjugants. Transconjugants were grown overnight on NA amended with 20 mg L^{-1} of copper for induction of resistance to copper (Basim et al., 2005) and suspended in sterile tap water visually to approximately 10^8 cfu mL^{-1}. Suspensions were then spotted on NA amended with 200 mg L^{-1} of copper to screen for clones carrying copper resistance genes.

General DNA manipulations

Miniscale preparations of *E. coli* plasmid DNA were obtained by alkaline lysis as described by Sambrook et al. (1989). Subcloning of the DNA insert from a cosmid carrying the copper resistance gene cluster was performed by restriction digestion of the original clone with various enzymes and purification of fragments from an agarose gel by using the Wizard PCR Preps DNA purification system (Promega, Madison, WI). Fragments were ligated into pBluescript II/KS (Stratagene, La Jolla, CA) and pLAFR3 (Staskawicz et al., 1987) vectors for nucleotide sequencing and for checking for copper resistance activity in ME24 by triparental mating as aforementioned. Ligation was performed with T4 DNA ligase (Promega, Madison, WI) used according to the manufacturer's instructions. Ligation products were transformed into competent cells of *E. coli* DH5α produced by the calcium chloride procedure as described by Sambrook et al. (1989).

Transposon mutagenesis of copper resistance genes from *Xanthomonas citri* subsp. *citri*

Mutagenesis was performed by randomly inserting Tn3-*uidA* transposon as previously described (Bonas et al., 1989) into pXccCu2 from Xcc A44 to assess for genes involved in copper resistance. Individual insertion derivatives were analyzed by extracting plasmid DNA and sequencing for location of transposon insertion within the

9.5 kb cloned fragment carrying copper resistance genes. Selected pXccCu2 derivatives were transferred to the recipient strain Xp 91-118 resistant to rifamycin and spectinomycin through triparental mating as described previously. To assess for copper resistance, transconjugants were grown overnight on NA amended with 20 mg L^{-1} of copper for induction of resistance (Basim et al., 2005), suspended in sterile tap water at approximately 10^8 cfu mL^{-1}, and then spotted (10 µL) on MGY agar (Cooksey and Azad, 1992; Bender et al., 1990) amended with 0, 25, 50, 100, 150, 200, 300, 400, 600 and 800 mg L^{-1} of copper. Growth of transconjugants was assessed after 96 h of incubation at 28 ^{o}C.

Design of primers for copper resistance genes and PCR analysis

Custom primers were designed to specifically amplify partial nucleotide sequences of copper resistance genes *copL*, *copA* and *copB* from Xcc using PCR (Polymerase Chain Reaction) analysis. Due to the presence of homologous genes in the chromosome of copper sensitive (Cu^S) strains of Xcc, nucleotide sequences of *copL*, *copA* and *copB* from Xcc A44 were aligned with chromosomal homologous ORFs of these genes (ORFXAC3629, ORF XAC3630, and ORF XAC3631, respectively) from Cu^S Xcc 306 (da Silva et al., 2002) using Clustal W (Thompson et al., 1994). Primer sets were designed based on regions of low or no similarity between resistant and sensitive strains to specifically amplify Cu^R strains. Bacterial strains tested for the presence of copper resistance genes using the newly designed primers are listed in Table 4-2.

Primers were synthesized by Sigma-Aldrich (Sigma-Aldrich Co., St. Louis, MO). Amplification of target genes from all bacteria was performed using a DNA thermal cycler (MJ Research PTC 100, Cambridge, MA) and the Taq polymerase kit (Promega,

Madison, WI). For extraction of template DNA, strains were individually grown overnight on NA, suspended in sterile deionized water (DI), boiled for 15 min, cooled on ice for 5 min, centrifuged at 15,000 rpm for 5 min and kept on ice. The supernatant was used for PCR reactions. Each PCR reaction mixture consisted of 25 µL total volume, which included10.3 µL of sterile water, 5 µL of 5 × PCR buffer, 1.5 µL of 25 mM $MgCl_2$, 4 µL deoxyribonucleoside triphosphates (0.8 mM each dATP, dTTP, dGTP, and dCTP), 0.5 µL of each primer (stock concentration, 25 pmol μL^{-1}), 3 µL of template, and 0.2 µL (5 U/µl) of Taq DNA polymerase. PCR reactions were initially incubated at 95°C for 5 min then 30 PCR cycles, which were run under the following conditions: denaturation at 95°C for 30 s, primer annealing at 60^{o}C for all set of primers for 30 s, and DNA extension at 72°C for 45 s in each cycle. After the last cycle, PCR tubes were incubated for 10 min at 72°C and then at 4°C. Cu^{R} Xcc A44 and Cu^{S} Xcc 306 were used as positive and negative controls, respectively. PCR reaction mixtures were analyzed by 2% agarose gel electrophoresis (Bio-Rad Laboratories, Hercules, CA) with Tris-acetate-EDTA (TAE) buffer system. A 50-bp DNA ladder (Promega, Madison, WI) was used as the standard molecular size marker for PCR product sizing. Reaction products were visualized by staining the gel with ethidium bromide (0.5 µg mL^{-1}) for 20 min and then photographed using a UV transilluminator and Quantity One software (Bio-Rad Universal Hood II, Hercules, CA).

Complete sequences of *copB* from *Xanthomonas* strains showing different sizes for this gene were PCR amplified using primers copBBF (5'- AGGTAGCCGACGCACG TATC – 3') and copBBR (5' – CCACCGCAACCAATGCCACG – 3'), designed based on the pXccCu2 sequence. Amplification of *copF* from Xcc A44 was performed by using

primers copFF (5’ – GCCCTGTTCCAGAGCACCTACGG – 3’) and copFR (5’ – CCTTGTTGGCATCGAGCTTGGTG – 3’) designed based on sequences from *Stenotrophomonas maltophilia* K279a (Stm K279a) (Crossman et al., 2008). These PCR reactions were performed as previously described and analyzed by 1% agarose gel electrophoresis. Lambda DNA digested with HindIII and EcoRI (Promega, Madison, WI) was used as molecular size marker.

DNA sequencing

DNA sequencing was performed by the DNA Sequencing Core Laboratory of the Interdisciplinary Center for Biotechnology Research (ICBR), University of Florida, Gainesville. For sequence analysis, DNA fragments were cloned into the vector pBluescript II/KS (Stratagene, La Jolla, CA) using appropriate enzymes. Sequencing was initiated using the standard flanking vector F20 and R24 primers. Custom primers designed based on the sequences obtained with F20 and R24 primers were used to complete the sequencing. The exact location of Tn3-*uidA* insertions was determined by sequencing plasmid DNA from insertion derivatives using primer RST92 (5’ - GATTTCACGGGTTGGGGTT TCT - 3’) which is complementary to the N-terminal of the transposon. Sequencing of PCR products of *copL*, *copA*, *copB* and *copF* was performed with primers used for PCR analysis. Additional custom primers designed based on sequences obtained with PCR primers were utilized for the complete sequencing of *copF*.

Comparison of copper resistance genes

Nucleotide sequences of clones carrying Cu^R genes from Xcc A44 and Xac 1381 were analyzed in Genbank using Basic Local Alignment Search Tool for Nucleotides (BLASTN), National Center for Biotechnology Information (NCBI) (Altschul et al, 1997).

Sequences of copper resistance genes *copL*, *copA* and *copB* from Cu^R strains (Table 4-6) generated in this study and from other strains previously sequenced, such as Stm K279a (Crossman et al., 2008) and Xp 7882 (Voloudakis et al., 2005) were compared phylogenetically. Phylogenetic trees were constructed using the Molecular Evolutionary Genetics Analysis version 4.0 (MEGA 4) software suite (Tamura et al., 2007). Chromosomal homologues of *copL*, *copA* and *copB* (ORFXAC3629, ORF XAC3630, and ORF XAC3631, respectively) from Cu^S Xcc 306 (da Silva et al., 2002) were used as outgroups. Each set of sequences was aligned using the default settings of Clustal W (Thompson et al., 1994). The alignments were assembled into maximum parsimony trees using heuristic searches with random stepwise addition. Branch support for the maximum parsimony tree was estimated using nonparametric bootstrap analysis with 1,000 replicates (Efron et al., 1996; Felsenstein, 1985) and 70% branch cut-off (Hillis and Bull, 1993). The percentage of replicate trees in which the associated strains clustered together in the bootstrap test is shown next to the branches (Felsenstein, 1985).

Results

Cloning and subcloning of copper resistance genes from *Xanthomonas citri* subsp. *citri* and *Xanthomonas alfalfae* subsp. *citrumelonis*

Approximately 600 and 1,600 clones were screened for Xcc A44 and Xac 1381 genomic libraries, respectively. One cosmid clone from each genomic library conferred copper resistance to Cu^S Xp ME24 transconjugants. Different fragment sizes from the original clones were subcloned into pLAFR3 (Staskawicz et al., 1987) and checked for copper resistance. An EcoRI-EcoRI 9.5 kb subclone (pXccCu2) obtained from the ~17 kb Sau3AI- Sau3AI cosmid clone from Xcc A44 (pXccCu1) and an HindIII-EcoRI 9.6 kb

subclone (pXacCu2) from the ~17 kb Sau3AI- Sau3AI original clone (pXacCu1) from Xac 1381 conferred resistance to copper on media containing 200 mg L^{-1} of copper sulfate.

Sequence analysis of the copper resistance genes

The nucleotide sequences for the copper resistance genes from Xcc and Xac have been assigned accession numbers HM362782 and HM579937, respectively, by GenBank.

Ten ORFs were identified for the sequence of the 9.5 kb DNA insert of pXccCu2 from Xcc A44 (Figure 4-1). These ORFs are located within ~7.9 kb. No ORF was identified in 1.6 kb positioned upstream of the first ORF. Seven ORFs are closely related to copper resistance genes previously sequenced (Crossman et al., 2008; Voloudakis et al., 2005). ORF2, ORF3, ORF4, ORF5, ORF7, ORF9, and ORF10 are ≥96% identical to genes related to copper resistance, *copL*, *copA*, *copB*, *copM*, *copG*, *copC* and *copD*, respectively, from Stm K279a isolated from an immunosuppressed patient (Crossman et al., 2008) (Table 4-3; Figure 4-2A and B). Additionally, *copL*, *copA* and *copB* from Xcc A44 are ≥93% identical to the same *cop* genes from Xp 7882 (Xp 7882) (Voloudakis et al., 2005), which lacks *copC* and *copD* (Table 4-3; Figure 4-2A and B). *copM* and *copG* from Xcc A44 are not as similar to the homologs in Xp 7882 as Stm K279a (Table 4-3; Figure 4-2B). Identity of *copM* and *copG* between the Xcc and Xp strains is lower than 70% and 90%, respectively (Table 4-3).

Immediately downstream of *copD* and *copG* in Stm K279a and Xp 7882, respectively, there is an ORF named *copF* that is absent in pXccCu2 (Figure 4-2A), whose sequence, based on Stm K279a, ends 44 bp upstream of the last nucleotide of *copD*. PCR analysis of Xcc A44 using primers designed based on Stm K279a and

sequencing of PCR product revealed the existence of *copF* in Xcc A44 (ORF11) (Figure 4-1), which is highly similar (≥95%) to *copF* from Stm K279a and Xp 7882 (Table 4-3; Figure 4-2B). It also confirmed that the cloned fragment harboring the copper resistance determinants from Xcc A44 lacks the last 44 nucleotides of *copD*. ORF1, ORF6 and ORF8 from pXccCu2 are also present in Stm K279a and seem to be related to a hypothetical transcriptional repressor, a transposase and a hypothetical protein, respectively (Figure 4-1 and 4-2A). Only part of the C-terminal of ORF1 is present in Xp 7882 and ORF6 and ORF8 are absent in that strain (Voloudakis et al., 2005) (Figure 4-2A).

Seven ORFs were identified for the sequence of the 9.6 kb DNA insert of pXacCu2 from Xac 1381 (Figure 4-1). These ORFs are located within ~8.1 kb. No significant ORF was identified in 1.2 kb and 0.3 kb positioned upstream and downstream, respectively of the first ORF. All ORFs except ORF1 are related to copper resistance genes previously described for Stm K279a (Crossman et al., 2008) and Xp 7882 (Voloudakis et al., 2005). Copper resistance genes from Xac 1381 are closer related to Xp 7882, whereas Xcc A44 showed greater similarity with Stm K279a, (Table 4-3; Figure 4-2A and B). ORF2, ORF3, ORF4, ORF5, ORF6, and ORF7 from pXacCu2 are ≥94% identical to *copL*, *copA*, *copB*, *copM*, *copG*, and *copF* from Xp 7882 (Table 4-3; Figure 4-2B). There is high sequence identity (≥92%) between *copL*, *copA*, *copB* and *copF* from Xcc 1381 and Xcc A44, Xp 7882, and Stm K279a (Table 4-3; Figure 4-2B). However, in Xac 1381 and Xp 7882 *copC* and *copD* are absent and nucleotide sequences of *copM* and *copG* are not as identical to the homologues in Xcc A44 and Stm K279a (Table 4-3; Figure 4-2B). ORFs related to a transposase and a hypothetical protein present in pXccCu2 from

XccA44 are absent in pXacCu2 from Xcc1381 and as for Xcc A44, ORF1 from Xac 1381 has higher homology to a hypothetical transcriptional repressor (Figures 4-1 and 4-2A).

The cluster *copLAB* is the most conserved region among the strains Xcc A44, Xac 1381, Xp 7882 and Stm K279a (Figures 4-2 to 4-8). The identity of these genes among the strains ranges from 92 to 99%. These copper resistance genes are presumptively located on plasmids. Homologues of these plasmid-borne copper resistance genes are present on the chromosome of copper sensitive and resistant *Xanthomonas* strains and display the same organizational pattern observed for the resistance genes from Cu^R strains A44, Xac 1381, Xp 7882 and Stm K279a (Figures 4-2A and C). However, on the chromosome no other homolog or additional gene is present downstream of *copB* (Figure 4-2C). Homology level between chromosomal and plasmid-borne genes is higher for *copA* and copB in comparison to *copL*. Copper resistance genes *copA* and *copB* are approximately 50 to 75% similar to the chromosomal homologs from strains Xcc 306 (da Silva et al., 2002) and Xv 85-10 (Thieme et al., 2005), which are known to be copper sensitive strains. In contrast, similarity of *copL* from Xcc A44 to Xcc 306 and 85-10 is lower than 40%.

PCR analysis of strains

PCR products were specifically amplified from Cu^R strains (Table 4-2) using primers designed based on nucleotide sequences of *copL*, *copA* and *copB* from Xcc A44 (Table 4-4). According to PCR analysis, these three genes are conserved in Cu^R strains of Xcc, Xac, Xv, Xp, Xaj, *X. euvesicatoria*, *X. gardneri*, epiphytic *X.* sp. and *S. maltophilia.* No amplification was detected for Cu^S strains.

Three different sizes for *copB* were observed in the PCR amplification of partial sequences of this gene. Most of the strains analyzed have an intermediate *copB* size. Based on PCR amplification of partial sequence of *copB*, of the 37 Cu^R strains included in this study (Table 4-2) 1 (3%), 9 (24%) and 27 (73%) strains were larger, smaller and intermediate in size. No differences in size were observed for *copL* and *copA* for the strains based on partial gene sequences analyzed.

Comparison of *copB* sequences in copper-resistant xanthomonads.

Xcc A44, Xe 81-23 and a pathogenic *Xanthomonas* with no defined host (Xsp 1219) were selected as representative strains for intermediate, small and large size *copB* sequences, respectively, and compared regarding the nucleotide sequence of the complete gene. Sequence analysis revealed that *copB* is 1269 bp, 1158 bp and 1425 bp in length in Xcc A44, Xe 81-23 and Xsp 1219, respectively (Figure 4-9). The alignment of nucleotide sequences of the three *copB* sizes demonstrates that the shorter sequence length in Xcc A44 and Xe 81-23 are due to a major nucleotide gap present in the same region of the gene in comparison to Xsp 1219 (Figure 4-9). In Xcc A44 there is a 153 bp gap from position 414 to 567 in Xsp 1219 (Figure 4-9). The same gap in Xe 81-23 is 300 bp long ranging from nucleotide position 375 to 674 in Xsp 1219 (Figure 4-9). Other differences include a 36 bp gap in Xcc A44 and Xsp 1219 in comparison to Xe 81-23 and an extra codon in the beginning of the N-terminal of *copB* in Xsp 1219 in comparison with the other two strains (Figure 4-9). All gaps observed when comparing the nucleotide sequence of *copB* for these strains were in frame (Figure 4-9).

Transposon mutagenesis of copper resistance genes from Xanthomonas citri subsp. citri

Transposon mutagenesis of cloned copper resistance determinants in Xcc A44 revealed that *copL*, *copA* and *copB* are the most important genes for copper resistance in Xcc. Transconjugant Xp 91-118 strains carrying mutated pXccCu2 was plated on MGY agar supplemented with different concentrations of copper. Mutation of *copL* and *copA* lowered copper resistance to levels tolerated by copper sensitive strains. Irrespective of the mutation site in the genes *copL*, *copA* and *copB*, mutants had resistance reduced to 50, 50 and 75 mg L^{-1} of copper, respectively (Table 4-5; Figure 4-10). As a reference, transconjugant 91-118 harboring pXccCu2 or pXacCu2 can resist up to 300 mg L^{-1} of copper on MGY and WT Xcc A44 and Xac 1381 are able to grow on MGY supplemented with 400 mg L^{-1} of copper. Mutations in the N-terminal region of *copM* that shares high homology with other copper resistant strains, such as Xac 1381, Xp 7882 and Stm K279a, reduced copper resistance slightly and mutants were able to grow up to 200 mg L^{-1} of Cu (Table 4-5; Figure 4-10). Irrespective of the insertion site in the gene, no change in copper resistance was observed when the transposon was inserted in *copG*, *copC*, and *copD* or in the ORF1, which homolog to a transcriptional repressor and is located right before of *copL* (Table 4-5; Figure 4-10). Insertional mutations of the region upstream of ORF1 did not affect resistance to copper (Table 4-5; Figure 4-10).

Phylogenetic analysis of copper resistance genes

Partial sequences of *copL*, *copA*, and *copB* obtained from different strains (Table 4-2) were used for phylogenetic analysis. Accession numbers assigned by GenBank for these sequences are listed in Table 4-6. Sequence alignment of PCR products revealed

high homology (>90%) for *copL*, *copA* and *copB* among different species and/or strains of *Xanthomonas* and *Stenotrophomonas*. Slight differences were discerned through phylogenetic analysis, which separated or grouped the strains based on the conservation of nucleotide sequences of these genes. Strain grouping showed consistency and the same branching pattern was observed for the three genes. However, such a pattern was not based on species or geographical origin. Phylogenetic analysis grouped together strains of different species from different countries around the world (Figures 4-11 to 13).

cop genes in Xac were more diverse than in Xcc. Of five Xcc strains, all from Argentina, four were always branched together with other strains of Xe, Xg, and Xv from Costa Rica and Xe from Guadeloupe (Figures 4-11 to 13). The remaining one, Xcc AR79, was grouped differently with other strains, including BV5-4, a strain of Xv isolated from Argentina as well (Figures 4-11 to 13). Conversely, the five Xac strains were placed into three different groups with other species (Figures 4-11 to 13).

Discussion

This is the first time copper resistance has been characterized in Xcc and Xac strains. We identified the determinants for copper resistance on a 7.9 kp EcoRI-EcoRI fragment in Xcc strain A44 from Argentina and a 8.1 kb EcoRI-HindIII in Xac strain 1381 from Florida. As a result of sequencing of these fragments ten and seven ORFs were identified in the cluster of genes associated with copper resistance in Xcc and Xac, respectively. In Xcc, ORF2, ORF3 and ORF4 were required for a high level of resistance in transconjugant screening. These three ORFs have high homology with *copL*, *copA* and *copB*, respectively from Stm K279a (Crossman et al., 2008) and Xp 7882 (Voloudakis et al., 2005). Insertional mutation of ORF7, ORF8, ORF9, and

ORF10, which exhibit homology to the *copG*, *copC*, and *copD*, respectively from Stm K279a (Crossman et al., 2008) had no observable effect on copper resistance when tested in the Xp 91-118 transconjugant background. Likewise, mutation of ORF1, which is homologous to a hypothetical transcriptional repressor from several bacteria, did not affect copper resistance. Mutation of ORF5, which is homologous to *copM* (also referred as cytocrom c) from Stm K279a (Crossman et al., 2008) slightly reduced copper resistance of transconjugants.

Copper resistance of transconjugant strains of different Cu^S *Xanthomonas* species carrying pXccCu2, which harbors the copper determinants from Xcc, showed a slight reduction of resistance on MGY agar (from 400 to 300 mg L^{-1}) when compared to the WT strain Xcc A44. Such a decrease on resistance could be explained by the absence of *copF* and incompleteness of *copD* in pXccCu2. However, the fact that the same behavior was observed for WT Xac 1381 and its clone pXacCu2, which harbors all the same genes identified in pXccCu2 and *copL*, suggests either that *copF* is not important for resistance and the slight decrease of resistance was due to the fact that the cloned copper resistance determinants were expressed in a different strain or that other genes might be involved in full copper resistance. If the latter is correct, the presumptive additional gene(s) is likely to be located far from the cloned gene cluster. No other ORF related to copper resistance was found upstream of ORF1 in pXccCu2 and Stm K279a or downstream of *copF* in Stm K279a. As discussed earlier, the organization, size and nucleotide sequences of genes in pXccCu2 and Stm K279a, which belongs to the *Xanthomonadaceae* family as well, are highly similar, thus making Stm K279a a reliable reference. *S. maltophilia* is ubiquitous in aqueous environments, soil and plants,

including water, urine, or respiratory secretions and was grouped in the genus *Xanthomonas* before becoming the type species of the genus *Stenotrophomonas* (Palleroni and Bradbury, 1993).

Comparison of copper resistance determinants in Xcc A44, Xac 1381, Stm K279a (Crossman et al., 2008) and Xp 7882 (Voloudakis et al., 2005) revealed that high homology (≥92%) of nucleotide sequences is maintained among these strains only for *copLAB*, 70% of the N-terminal of *copM*, which is positioned immediately after *copB*, and *copF*, which is located at the end of the gene cluster in all strains. Although we could not determine the importance of *copF* for copper resistance by insertional mutation because this gene is absent in pXccCu2, we were able to demonstrate that the conserved region *copLAB* and part of *copM* has direct involvement in copper resistance. *copLAB* is essential for copper resistance and the N-terminal of *copM* is necessary for full resistance.

The individual function of the homologous genes identified in pXccCu2 and pXacCu2 for conferring copper resistance in *Xanthomonas* is not completely revealed. Besides CopL, which was demonstrated to be involved in regulation of copper resistance (Voloudakis et al., 2005), the putative role of the other genes has been presumed based on homologous genes from other organisms. It seems that CopA and CopB are copper binding proteins, CopM is a cytochrome C oxidase involved in electron transport, CopG is a hypothetical exported protein, CopC and copD are transmembrane transporter proteins, and CopF is a putative copper-transporting p-type ATPase (Crossman et al., 2008; Voloudakis et al., 2005).

Homologues of the copper resistance genes *copLAB* cloned from Xcc A44 and Xac 1381 are present on the chromosome of Cu^R strains, such as Xv 1111 (data not published), and strains that have been tested to be Cu^S, such as Xcc 306 (da Silva et al., 2002) and Xv 85-10 (Thieme et al., 2005). Homologues of these genes are also present in many other *Xanthomonas* strains, including *X. oryzae* pv. *oryzae*, *X.campestris* pv. *vesicatoria*, *X. campestris* pv. *campestris*, whose resistance or sensitivity to copper is unconfirmed. On the chromosome, the homologues display the same organizational pattern observed for the resistance genes from Cu^R strains, however no other ORF related to copper resistance is identified downstream of chromosomal *copLAB* in Xcc 306 and Xv 85-10, as demonstrated for the actual resistance genes from Xcc A44 and Xac1381.

The presence of homologues of copper resistance genes on the chromosome has been previously reported for other bacteria. Chromosomal genes that hybridize with the *cop* operon were detected in Cu^R and Cu^S strains of *Pseudomonas* (Cooksey et al., 1990). In *P. syringae*, *cop* homologues have been detected in more than 20 Cu^S strains from eight pathovars (Lim and Cooksey, 1993). Furthermore, it has been demonstrated that in several strains of *P. syringe* these chromosomal homologues can activate the plasmid-borne *cop* promoter (Lim and Cooksey, 1993; Mills et a., 1993), reflecting a possible chromosomal origin of the plasmid-borne resistance genes.

Differently from what has been annotated, chromosomal *copLAB is* not responsible for copper resistance, but likely necessary for homeostasis and/or tolerance. Teixeira et al. (2008) demonstrated that chromosomal *copAB* from Xcc 306 is responsive to copper amendments, however this strain was mistakenly rated as copper

resistant. This was probably due to pH adjustments made to medium with potassium phosphate buffer, which chelates copper ions and changes the actual concentration of copper available in the medium (Teixeira et al., 2008). While strains harboring the copper resistance genes *copLAB* highly similar (≥90%) to the ones cloned in this study can grow on MGY agar amended up to 400 mg L^{-1} of Cu, strains that have only the chromosomal *copLAB* genes, such as Xcc 306, grow up to 75 mg L^{-1} of Cu, hence, are Cu^S. Thus, to avoid further confusion or misinterpretation we suggest that the nomenclature of chromosomal homologues of *copL*, *copA* and *copB* in xanthomonads, which are probably copper homeostasis genes, should be changed to *cohL*, *cohA* and *cohB*, respectively.

Sequence alignments of *copLAB* genes from different strains and/or species of *Xanthomonas* indicated that the resistance genes are conserved among the Cu^R strains with identity of nucleotide sequences higher than 90%. Phylogenetic analysis revealed that the minor differences which exist in the nucleotide sequences of these strains are not related to the species or geographical origin. Xcc strains from Argentina were clustered into two different groups. Four strains were more closely related to strains of Xe, Xg and Xv from Costa Rica and Guadeloupe, and one Xcc strain was associated with an Xv strain also isolated from Argentina. This indicates that the copper resistance in xanthomonads may have a common origin and that the Cu^R genes have been independently exchanged among different species of xanthomonads, possibly by horizontal transfer. The presence of copper resistance genes from plant pathogenic xanthomonads in epiphytic bacteria such as *Xanthomonas* and *Stenotrophomonas,* as demonstrated in this study and the incessant movement of plant material, especially

seeds, among countries may account for such wide dissemination of these genes into different *Xanthomonas* populations in different parts of the world, indicating a relatively high risk for copper resistance development in *Xanthomonas* pathogens under constant exposure to copper.

Table 4-1. Bacterial strains and plasmids used in this study.

Strain or plasmid	Relevant characteristic	Reference or source
Escherichia coli		
DH5α	F80d *lacZ*15 *recA1*	GIBCO-BRL
C2110	Nal^R *polA*	Bonas et al., 1991
Xanthomonas citri subsp. citri		
A44	Cu^R	Canteros, 1996
Xanthomonas alfalfae subsp. citrumelonis		
1381	Cu^R	This study
Xanthomonas perforans		
91-118	Kan^R	
Plasmids		
pLAFR3	Tet^R, rlx^+, RK2 replicon	Staskawicz et al., 1987
pBluescript KS+/-	Phagemid, pUC derivative, Amp^R	Stratagene
pRK2073	ColEI replicon, Tra^+ Mob^+, Sp^R	Figurski and Helinski, 1979
pXccCu1	Tet^R, Cu^R, ~17 kb EcoRI-HindII fragment of Xcc A44 in pLAFR3	This study
pXccCu2	Tet^R, Cu^R, 9.5 kb EcoRI- EcoRI fragment of pXccCu1	This study
pXacCu1	Tet^R, Cu^R, ~17 kb EcoRI-HindII fragment of Xac 1381 in pLAFR3	This study
pXacCu2	Tet^R, Cu^R, 9.6 kb HindIII- EcoRI fragment of pXacCu1	This study

Table 4-2. Bacterial strains tested for the presence of copper resistance genes through PCR analysis using primers designed based on *copL*, *copA*, and *copB* genes from *Xanthomonas citri* subsp. *citri* A44.

Organism [a]	Strain	copper resistant [a]	Geographical origin	Year of isolation	Source [d]
Xcc	306	N	Brazil	1997	da Silva et al., 2002
Xcc	FB06C	N	Florida	2007	This study
Xcc	AR78	N	Argentina	2008	This study
Xcc	AR81	N	Argentina	2008	This study
Xcc	A44	Y	Argentina	1994	Canteros, 1996
Xcc	AR63	Y	Argentina	2008	This study
Xcc	AR72	Y	Argentina	2008	This study
Xcc	AR77	Y	Argentina	2008	This study
Xcc	AR79	Y	Argentina	2008	This study
Xac	1390	N	Florida	2000	DPI[c]
Xac	1381	Y	Florida	2000	DPI
Xac	1382	Y	Florida	2000	DPI
Xac	1383	Y	Florida	2000	DPI
Xac	7589	Y	Florida	2007	DPI
Xac	9226	Y	Florida	2007	DPI
Xac	29354	Y	Florida	2009	DPI
Xv	82-8	N	Florida	1982	Stall, R. E.
Xv	1111	Y	New Zealand	1955	ATCC
Xv	BV5-4	Y	Argentina	1987	Canteros, B.I.
Xv	Xv56	Y	Brazil	nd[d]	nd
Xv	Xv446	Y	Costa Rica	1991	Jones, J. B.
Xv	Xv1288	Y	Michigan	nd	Jones, J. B.
Xsp	1219	Y	Ohio	1995	Jones, J. B.
Xe	81-23	Y	Florida	1981	Stall, R. E.
Xe	E-3	Y	Florida	1960	Stall, R. E.
Xe	75-3	Y	Florida	1975	Stall, R. E.
Xe	Xv221	Y	Guadeloupe	1990	Jones, J. B.
Xe	Xv264	Y	Guadeloupe	1990	Jones, J. B.
Xe	Xv304	Y	Barbados	1990	Jones, J. B.
Xe	Xv477	Y	Costa Rica	1991	Jones, J. B.
Xe	Xv669	Y	Puerto Rico	1991	Jones, J. B.
Xe	Xv718	Y	Puerto Rico	1991	Jones, J. B.
Xe	Xv787	Y	US Virgin Islands	1991	Jones, J. B.

Table 4-2. Continued

Xe	Xv800	Y	US Virgin Islands	1991	Jones, J. B.
Xe	Xv818	Y	Spain	nd	Jones, J. B.
Xe	Xv881	Y	Mexico	1992	Jones, J. B.
Xe	Xv1025	Y	Mexico	1992	Jones, J. B.
Xp	91-118	N	Florida	1991	Stall, R. E.
Xp	1-7	Y	Florida	2006	Stall, R. E.
Xg	Xv444	Y	Costa Rica	1991	Jones, J. B.
Xaj	Xj71	Y	Italy	2008	Dallai, D.
Xaj	Xj79	Y	Italy	2008	Dallai, D.
Xsp	INA69	Y	Florida	1984	Minsavage, G. V.
Stm	FB03P	Y	Florida	2008	This Study

[a] Xcc, *Xanthomonas citri* subsp. *citri*; Xac, *Xanthomonas alfalfae* subsp. *citrumelonis*; Xv, *Xanthomonas vesicatoria*; Xe, *Xanthomonas euvesicatoria*; Xp, *Xanthomonas perforans*; ; Xp, *Xanthomonas gardneri*; Xaj, *Xanthomonas arboricola* pv. *juglandis*; Xsp, *Xanthomonas* sp.(1219, pathogenic; INA69, non-pathogenic); Stm, *Stenotrophomonas maltophilia;*

[b] N, no; Y, yes;

[c] DPI, Division of Plant Industry, Department of Agriculture and Consumer Services, Gainesville, FL; ATCC, American Type Culture Collection; Canteros, B.I., Instituto Nacional de Tecnología Agropecuaria, Bella Vista, Argentina; Dallai, D., University of Modena & Reggio Emilia, Reggio Emilia, Italy; Stall, R.E., University of Florida, Gainesville, FL; Jones, J.B., University of Florida, Gainesville, FL; Minsavage, G.V., University of Florida, Gainesville, FL.

[d] nd, not determined.

Table 4-3. Comparison of nucleotide sequences of genes *copL*, *copA*, *copB*, *copM*, *copG*, *copC, copD*, and *copF* from different strains.

Organism/Strain [a]	Xcc A44	Xac 1381	Xp 7882
Xcc A44	-	-	-
Xac 1381	92 (100)[b]	-	-
Xp 7882	93 (100)	96 (100)	-
Stm K279a	96 (100)	94 (100)	95 (100)
Xcc A44	-	-	-
Xac 1381	95 (100)	-	-
Xp 7882	95 (100)	97 (100)	-
Stm K279a	97 (100)	95 (100)	95 (100)
Xcc A44	-	-	-
Xac 1381	92 (100)	-	-
Xp 7882	93 (100)	94 (100)	-
Stm K279a	99 (100)	92 (100)	
Xcc A44	-	-	-
Xac 1381	91 (75)	-	-
Xp 7882	89 (75)	94 (100)	-
Stm K279a	99 (100)	91 (69)	89 (75)
Xcc A44	-	-	-
Xac 1381	69 (52)	-	-
Xp 7882	68 (55)	96 (100)	-
Stm K279a	100 (100)	69 (70)	68 (55)
Xcc A44	-	-	-
Xac 1381	nc[c]	-	-
Xp 7882	nc	nc	-
Stm K279a	99 (100)	nc	nc
Xcc A44	-	-	-
Xac 1381	nc	-	-
Xp 7882	nc	nc	-
Stm K279a	98 (100)	nc	nc
Xcc A44	-	-	-
Xac 1381	97 (97)	-	-
Xp 7882	95 (100)	94 (100)	-
Stm K279a	99 (100)	97 (92)	95 (97)

[a] Xcc, *Xanthomonas citri* subsp. *citri*; Xac, *Xanthomonas alfalfae* subsp. *citrumelonis*; Xp, *Xanthomonas perforans*; Stm, *Stenotrophomonas maltophilia*.

[b] numbers indicate identity and coverage of comparable sequence as % in parenthesis, respectively.

[c] nc, not comparable due to the absence of the gene in one or both strains

Table 4-4. Oligonucleotide primer sets used for screening for the presence of copper resistance genes *copL*, *copA* and *copB*.

Gene	Primer name	Sequence (5' to 3')	Length (bp[a])	GC[b] (%)	Tm[c] (^{o}C)	Product size (bp)
copL	copLF	CCGTGTCAAGCCTCCTCACTTCTAC	25	56	63	~360
	copLR	CAGCGGCATGACATCCAGGCC	21	67	63	
copA	copAF	CCTCCATGGCACGGACACTTCCATC	25	60	65	~870
	copAR	CCAGACATATCCATCGACCCATGATCCA	28	50	63	
copB	copBF	CTCAGGATCACTCTGCACATCAG	23	52	60	~535
	copBR	GCACGTAGCTCTTAATCGAGTTGTC	25	48	60	

[a] bp, base pair;[b] guanine (G) and cytosine (C) content; [c] calculated melting temperature

Table 4-5. Site of transposon insertion of selected derivatives and respective resistance to copper.

Mutant	Region mutated	Mutation site in the gene (bp)[a]	Gene size (bp)	Portion deleted (%)	Resistance to copper (mg L^{-1})[b]
M60	upstream of *cop* genes	1029 upstream of *copL*	-	-	300
M114	upstream of *cop* genes	717 upstream of *copL*	-	-	300
M257	hypothetical repressor	16	327	95	300
M357	*copL*	26	420	94	50
M377	*copL*	401	420	5	50
M206	between *copL* and *copA*	2 upstream of *copA*	-	-	50
M122	*copA*	659	1872	65	50
M08	*copA*	788	1872	58	50
M06	*copA*	1190	1872	36	50
M167	*copA*	1389	1872	26	50
M125	*copA*	1821	1872	3	50
M169	*copB*	113	1269	91	75
M160	*copB*	376	1269	70	75
M46	*copB*	647	1269	49	75
M10	*copB*	817	1269	36	75
M120	*copM*	90	771	88	200
M48	*copM*	138	771	82	200
M149	*copG*	67	525	87	300
M155	*copG*	271	525	48	300
M89	*copG*	505	525	4	300
M159	*copC*	341	384	11	300
M101	*copD*	194	930	79	300
M98	*copD*	686	930	26	300

[a] bp, base pair;
[b] maximum tolerated concentration of copper, as copper sulfate pentahydrate, amended to mannitol-glutamate yeast extract (MGY) agar.

Table 4-6. Accession numbers assigned by GenBank for partial sequences of *copL*, *copA*, and *copB* obtained from different strains.

Organism[a]	Strain	Gene		
		copL	*copA*	*copB*
Xac	9226	HM623134	HM626518	HM626553
Xac	7589	HM623135	HM626519	HM6265
Xac	29354	HM623136	HM626520	HM6265
Xcc	AR63	HM623137	HM626521	HM6265
Xcc	AR77	HM623138	HM626522	HM6265
Xcc	AR79	HM623139	HM626523	HM6265
Xcc	AR82	HM623140	HM626524	HM6265
Xe	81-23	HM623141	HM626525	HM6265
Xe	E-3	HM623142	HM626526	HM6265
Xv	1111	HM623143	HM626527	HM6265
Xe	75-3	HM623144	HM626528	HM6265
Xv	BV5-4	HM623145	HM626529	HM6265
Xp	1-7	HM623146	HM626530	HM6265
Xg	Xv444	HM623147	HM626531	HM6265
Xsp	INA69	HM623148	HM626532	HM6265
Stm	FB03P	HM623149	HM626533	HM6265
Xaj	71	HM623150	HM626534	HM6265
Xaj	79	HM623151	HM626535	HM6265
Xe	Xv669	HM623152	HM626536	HM6265
Xac	1382	HM623153	HM626537	HM6265
Xe	Xv718	HM623154	HM626538	HM6265
Xv	Xv446	HM623155	HM626539	HM6265
Xe	Xv881	HM623156	HM626540	HM6265
Xe	Xv264	HM623157	HM626541	HM6265
Xe	Xv304	HM623158	HM626542	HM6265
Xac	1383	HM623159	HM626543	HM6265
Xe	Xv787	HM623160	HM626544	HM6265
Xe	Xv800	HM623161	HM626545	HM6265
Xsp	1219	HM623162	HM626546	HM6265
Xe	Xv818	HM623163	HM626547	HM6265
Xv	Xv1188	HM623164	HM626548	HM6265
Xe	Xv1025	HM623165	HM626549	HM6265
Xe	Xv221	HM623166	HM626550	HM6265
Xe	Xv447	HM623167	HM626551	HM6265
Xv	Xv56	HM623168	HM626552	HM6265

[a] Xcc, *Xanthomonas citri* subsp. *citri*; Xac, *X. alfalfae* subsp. *citrumelonis*; Xv, *X. vesicatoria*; Xe, *X. euvesicatoria*; Xp, *X. perforans*; ; Xp, *X. gardneri*; Xaj, *X. arboricola* pv. *juglandis*; Xsp, *X.* sp.(1219, pathogenic; INA69, non-pathogenic); Stm, *Stenotrophomonas maltophilia.*

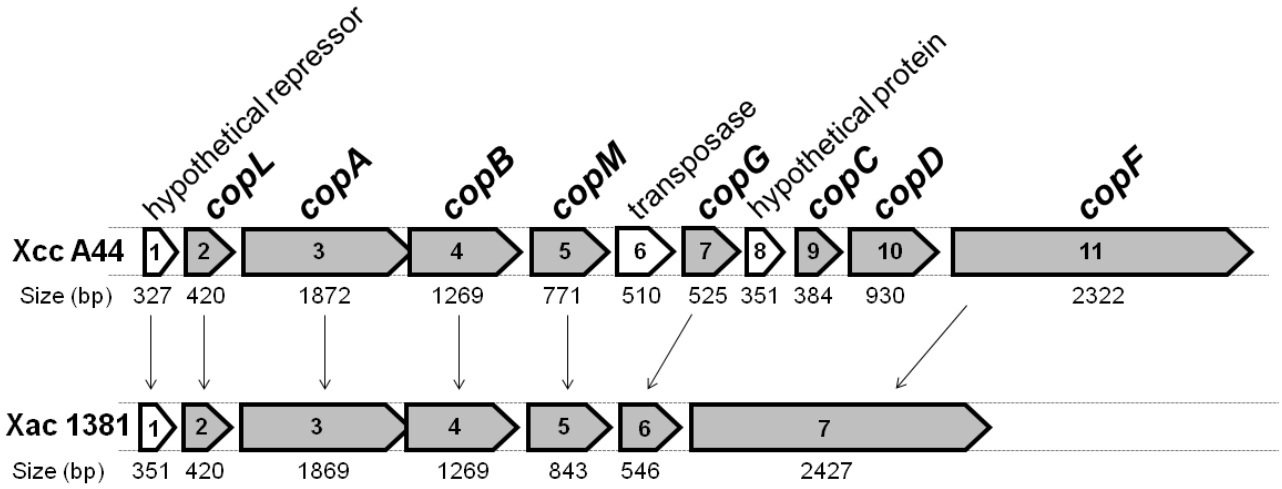

Figure 4-1. Copper resistance determinants in *Xanthomonas citri* subsp. *citri* (Xcc) strain A44 and *Xanthomonas alfalfae* subsp. *citrumelonis* (Xac) strain 1381. ORF number is indicated inside the shapes.

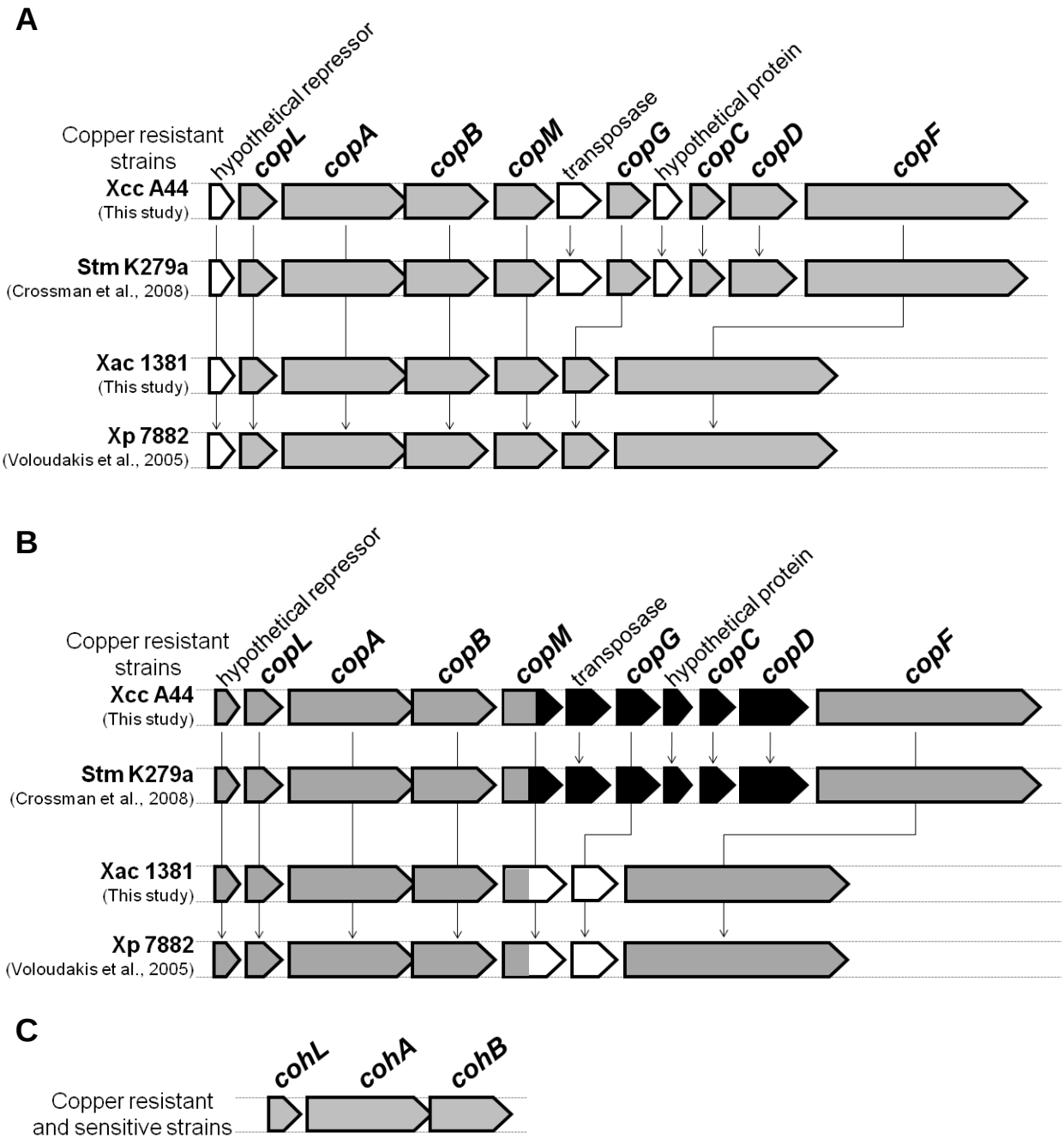

Figure 4-2. Comparison of genes involved in copper metabolism. A) comparison of different bacterial strains regarding the composition of the copper resistance gene cluster, B) comparison of copper resistance gene cluster regarding the identity of nucleotide sequences. Areas with the same color indicate conservation of nucleotide sequence among the strains with identity ≥92%, C) chromosomal genes homolog to *copL*, *copA* and *copB*, respectively, which are present in both copper sensitive and resistant strains of *Xanthomonas*. Xcc , *Xanthomonas citri* subsp. *citri*; Stm, *Stenotrophomonas maltophilia*; Xac, *Xanthomonas alfalfae* subsp. *citrumelonis*; Xp, *Xanthomonas perforans*.

```
XccA44       ATGTCGCCCGTGTCAGCCTCCTCACTTCTACTGCGACTGCTCCTGATCGTCATGCTCGTG 60
StmK279a     ATGTCGCCCGTGTCAGCCTCCTCACTTCTACTGCGACTGTTCCTGATCGCCATGCTCGTG 60
Xac1381      ATGTCGCCCGTGTCAGCCTCCTCACTTCTACTGCGACTGCTCCTGATCGCCATGCTCGTG 60
Xp7882       ATGTCGCCCGTGTCAGCCTCCTCACTTCTACTGCGACTGTTCCTGATCGCCATGCTCGTG 60
             ************************************ ********* **********

XccA44       CTTAACGGCGCGTGGTCGGCATTTGCGTCGATCAGTATGAACCCGGCCATGGAAGCGCAG 120
StmK279a     CTTAACGGCGCGTGGTCGGCGTTTGCGTCCGTCAGTATGAACCCGGTCATGGAAGAGCAG 120
Xac1381      CTTAACGGGGCGTGGTCGGCGTTTGCGTCGGTCAGTATGAATCCGGTGATGGAAGAGCAG 120
Xp7882       CTTAACGGGGCGTGGTCGGCGTTTGCGTCGGTCAGTATGAATCCGGTGATGGAAGAGCAG 120
             ******** *********** ********  ********** ****  ******* ****

XccA44       GCCAGCGAAGTGGCTGCCGCGGTGCAAGGCGACGAAGACTGCGTCGCCCATCACAGTGCT 180
StmK279a     GCCAGCGAAGTGGCTGCCGCGGTGCAAGTCGACGAAGACTGCTTCGCCCATCACAGTGCT 180
Xac1381      GCCAGTGAAGTGGCTGCCGCTGTGCAAGTCGACGAGGACTGCGTCGCCCATCACAGTGCT 180
Xp7882       GCCAGCGAAGTCGCTGCCGCGGTGCAAGTCGACGAAGACTGCGTCGCCCATCACAGTGCT 180
             ***** ***** ******** ******* ****** ****** ****************

XccA44       GAGAATCATCCCGATGCCACCTCGATTGAAAGGGCTGGCACTGGGCATGGCGACCATGCC 240
StmK279a     GAGCATCATCCCGATGCCACATCGATTGAAAAGGCTGGCACTGGGCATGGCGACCATGCC 240
Xac1381      GAGCATCATCCCGATGCCACATCGATTGAAATGGCTGGCACTGGGCACGGCGACCATGCC 240
Xp7882       GAGCATCATCCCGATGCCACATCGATTGAAAAGGCTGGCACTGGGCACGGCGATCATGCC 240
             *** ********************* ********** ********************** ***** ******

XccA44       GGTCCCGACTGTTGCAAGTCTTCTGCGTGCCGGTGCGCCTGCGTACACGCGTGCGCGAGC 300
StmK279a     GGTCCCGACTGTTGCAAGTCTTCTGCGTGCCGGTGCGCCTGCGTACACGCTTGCGCGAGC 300
Xac1381      GGTCCCGACTGTTGCAAGTCGTCTGCGTGCCGGTGCGCCTGCGTGCACGCGTGCGCGAGC 300
Xp7882       GGCCCCGACTGTTGCAAGTCGTCTGCGTGCCGGTGCGCCTGCGTGCACGCGTGCGCGAGC 300
             ** ********************* ********************** ***** *********

XccA44       GCACTTCCGGCGCGCCTGCATGTTTCGGTGCAACTGGCCTTGGGCCTGGATGTCATGCCG 360
StmK279a     GCACTTCCTGCGCGCCTGCATGTTTCGGTGCAACTGGCCTTGGGCCTGGATGTCATGCCG 360
Xac1381      GCATTGCCTGCGCGCCTGCATATTGCCGTACAACTGGCCTTGGGCCTGGATGTCATGCCG 360
Xp7882       GCATTGCCTGCGCGCCTGCATGTTTCGGTACAACTGGCCTTGGGCCTGGATGTCATGCCG 360
             *** * ** ************ ** * ** ****************************

XccA44       CTGCCCCAAGGGCATGCGGCACCTGCCTTGCCTCATCTGATCCGACCACCGATCGGCTAA 420
StmK279a     CTGCCCCTAGGGCATGCGGCACCTGCCTTGCCTCATCTGATCCGACCACCGATCGGCTAA 420
Xac1381      CTGCCGTTGGGGCATCCGGCACCCGCCTTGCCTCATCTGATCCGACCACCGATCGGCTAA 420
Xp7882       CTGCCCTTGGGGCATCCGGCGCCTGCCTTGCCTCATCTGATCCGACCACCGATCGGCTAA 420
             *****    ****** **** ** ********************************
```

Figure 4-3. Alignment of complete nucleotide sequences of *copL*. XccA44, *Xanthomonas citri* subsp. *citri* A44; StmK279a, *Stenotrophomonas maltophilia* K279a; Xac1381, *X. alfalfae* subsp. *citrumelonis* 1381; Xp7882, *X. perforans* 7882. The homology line indicates the degree of amino acid homology among all aligned sequence members. Asterisk (*), identical residue; gap, no conservation (Thompson et al., 1994).

```
Xp7882          MSPVSASSLLLRLFLIAMLVLNGAWSAFASVSMNPVMEEQASEVAAAVQVDEDCVAHHSA 60
StmK279a        MSPVSASSLLLRLFLIAMLVLNGAWSAFASVSMNPVMEEQASEVAAAVQVDEDCFAHHSA 60
Xav1381         MSPVSASSLLLRLLLIAMLVLNGAWSAFASVSMNPVMEEQASEVAAAVQVDEDCVAHHSA 60
XccA44          MSPVSASSLLLRLLLIVMLVLNGAWSAFASISMNPAMEAQASEVAAAVQGDEDCVAHHSA 60
                ************:**.************:****.** ********** ****.*****

Xp7882          EHHPDATSIEKAGTGHGDHAGPDCCKSSACRCACVHACASALPARLHVSVQLALGLDVMP 120
StmK279a        EHHPDATSIEKAGTGHGDHAGPDCCKSSACRCACVHACASALPARLHVSVQLALGLDVMP 120
Xav1381         EHHPDATSIEMAGTGHGDHAGPDCCKSSACRCACVHACASALPARLHIAVQLALGLDVMP 120
XccA44          ENHPDATSIERAGTGHGDHAGPDCCKSSACRCACVHACASALPARLHVSVQLALGLDVMP 120
                *:******** ************************************::***********

Xp7882          LPLGHPAPALPHLIRPPIG- 139
StmK279a        LPLGHAAPALPHLIRPPIG- 139
Xav1381         LPLGHPAPALPHLIRPPIG- 139
XccA44          LPQGHAAPALPHLIRPPIG- 139
                ** **.*************
```

Figure 4-4. Alignment of complete amino acid sequences of *copL*. XccA44, *Xanthomonas citri* subsp. *citri* A44; StmK279a, *Stenotrophomonas maltophilia* K279a; Xac1381, *X. alfalfae* subsp. *citrumelonis* 1381; Xp7882, *X. perforans* 7882. The homology line indicates the degree of amino acid homology among all aligned sequence members. Asterisk (*), identical residue; colon (**:**), conserved substitution; period (**.**), semiconserved substitution; gap, no conservation (Thompson et al., 1994).

```
XccA44      ATGTCGCATGATGATTTTCGTGGTCCACACGGTGGACCGCCGCTGCTACCTTCGCGGCGG 60
StmK279a    ATGTCGCATGATGATTTTCGTGGTCCACGCGGTGGACCGC---TGCTGCCTTCGCGGCGG 57
Xac1381     ATGTCGCATGATGATTTTCGTGGTCCACACGGTGGACCGC---TGCTGCCTTCGAGGCGG 57
Xp7882      ATGTCGCATGATGATTTTCGTGGTCCACATGGTGGACCGC---TGCTGCCTTCGCGGCGG 57
            ****************************  **********   **** ****** *****

XccA44      CGATTTGTCCAGGGCTTGGCCTTGGGAGGCGCAGTCGCAGGATTAGGTTTCTGGCCCAAA 120
StmK279a    CGATTTGTCCAAGGCTTGGCCTTGGGAGGCGCAGTCGCAGGATTAGGTTTCTGGCCCAAA 117
Xac1381     CGATTTGTCCAGGGCTTGGCCTTGGGAGGCGCAGTCGCAGGATTAGGGTTCTGGCCCAAG 117
Xp7882      CGATTTGTCCAAGGCTTGGCCTTGGGAGGCGCAGTCGCAGGATTGGGGTTCTGGCCCAAA 117
            *********** ********************************** ** ***********

XccA44      GCCAGTTGGGCGCTCAAGGGCCCGGGACAACCCAACGTACTATCGGGCACCGAGTTTGAC 180
StmK279a    GCCAGTTGGGCGCTCAAGGGGCCGGGACAACCCAACGTACTATCGGGCACTGAGTTTGAC 177
Xac1381     GCCAGTTGGGCGCTCAAGGGCCCGGGACAAGCCAACGTACTGTCGGGCACCGAGTTCGAC 177
Xp7882      GCCAGTTGGGCGCTCAAGGGCCCCGGACAAGCCAACGTATTGTCGGGCACCGAGTTTGAC 177
            ******************** ** ****** ******** * ******** ***** ***

XccA44      CTAACCATCGGCGAGACGCCGATGAACTTCACCGGCAAGACCCGCACCGCGATCACCGTC 240
StmK279a    CTGACCATTGGCGAGACGCCGATGAACTTCACCGGCAAGACCCGCACCGCGATCACGGTC 237
Xac1381     CTGACCATCGGCGAGACGCCGATGAACTTCACCGGCAAGACCCGCACCGCGATCACGGTC 237
Xp7882      CTGACCATCGGCGAGACGCCGATGAACTTCACCGGCAAGACCCGCACCGCGATCACGGTC 237
            ** ***** ********************************************** ***

XccA44      AATGGGTCCGTTCCGGCGCCGTTGCTGCGGTGGCGGGAAGGCACCACGGTCAGCCTCCGT 300
StmK279a    AACGGGTCCGTTCCGGCGCCGTTGCTGCGGTGGCGGGAAGGCACCACGGTCAACTTGCGC 297
Xac1381     AACGGATCCGTTCCGGCGCCGTTGCTGCGATGGCGGGAAGGCACCACGGTCAACCTGCGT 297
Xp7882      AACGGGTCCGTTCCGGCGCCGTTGCTGCGGTGGCGGGAAGGCACCACGGTCAACCTGCGT 297
            ** ** ********************** ********************** * * **

XccA44      GTCTCTAATGCATTGCCGGCCAACTCCCTCCATGGCACGGACACTTCCATCCATTGGCAC 360
StmK279a    GTCTCCAATGCATTGCCCGCTAACTCCATCCATGGCGCGGACACCTCCATCCATTGGCAC 357
Xac1381     GTCTCCAATGCATTGCCGGCCAATTCCCTCCATGGCACGGACACTTCCATCCATTGGCAC 357
Xp7882      GTCTCCAATGCATTGCCGGCCAACTCCCTCCATGGCACGGATACTTCCATCCATTGGCAC 357
            ***** *********** ** ** *** ******** **** ** ***************

XccA44      GGCATCATTCTGCCGGCCAACATGGACGGCGTGCCGGGCCTGAGCTTTGACGGTATCGGA 420
StmK279a    GGCATCATTTTGCCGGCCAACATGGACGGCGTGCCGGGTCTGAGCTTTGACGGTATCGGA 417
Xac1381     GGCATCATTCTGCCGGCCAACATGGACGGCGTGCCGGGACTGAGCTTTGACGGCATCGGA 417
Xp7882      GGCATCATTCTGCCGGCCAACATGGACGGCGTGCCGGGCCTGAGCTTTGACGGCATCGGA 417
            ********* ************************** ************** ******

XccA44      CGTGGTGAGACCTACCACTATCGGTTCACCCTGCATCAGGGCGGCACCTACTGGTACCAC 480
StmK279a    CGTGGTGAGACCTACCACTACCGGTTCACCCTGCATCAGGGCGGAACCTACTGGTACCAC 477
Xac1381     CGTGGTGAGACCTACCACTACCGGTTCACCCTGCATCAGGGCGGCACCTACTGGTACCAC 477
Xp7882      CGTGGTGAGACCTACCACTATAGGTTCACCCTGCATCAGGGCGGAACCTACTGGTACCAC 477
            ********************  ********************** ***************

XccA44      AGCCACTCAGGATTCCAGGAACAAGCCGGGCTTTATGGGCCGATCGTGATCGATCCACTG 540
StmK279a    AGCCACTCAGGGTTCCAGGAACAAGCCGGGCTCTATGGCCCGATCGTCATCGACCCATTG 537
Xac1381     AGCCACTCAGGTTTCCAGGAACAAGCAGGGCTTTATGGACCGATCGTGATCGATCCACTG 537
Xp7882      AGCCACTCAGGTTTCCAGGAACAAGCTGGGCTTTATGGACCGATCGTGATCGATCCACTG 537
            *********** ************** ***** ***** ******** ***** *** **

XccA44      GAGCCAGAGCCTTTCAGCTTCGATCGCGACTACGTCGTGATGCTGAGCGATTGGACAGAC 600
StmK279a    GAGCCGGAGCCCTTCAGTTTCGATCGCGACTACGTCGTGATGCTGAGCGATTGGACAGAC 597
Xac1381     GAGCCAGAGCCTTTTAGCTTCGATCGCGACTACGTCGTGATGCTGAGCGATTGGACAGAC 597
Xp7882      GAGCCAGAGCCTTTCAGCTTCGATCGCGACTACGTCGTGATGCTGAGCGATTGGACAGAC 597
            ***** ***** ** ** ****************************************
```

Figure 4-5. Alignment of complete nucleotide sequences of *copA.* XccA44, *Xanthomonas citri* subsp. *citri* A44; StmK279a, *Stenotrophomonas maltophilia* K279a; Xac1381, *X. alfalfae* subsp. *citrumelonis* 1381; Xp7882, *X. perforans* 7882. The homology line indicates the degree of amino acid homology among all aligned sequence members.Asterisk (*), identical residue; gap, no conservation (Thompson et al., 1994).

```
XccA44      CTGGACCCGACGGCCCTGTTCGATCGTTTGAAGAAGATGCCGGGCCATGACAACTATTAC 660
StmK279a    CTGGACCCGACGGCCCTGTTCGATCGTTTGAAGAAGATGCCGGGCCATGACAATTACTAC 657
Xac1381     CTGGACCCGGCGGCCCTGTTCGATCGTTTGAAGAAGATGCCGGGCCATGACAATTACTAC 657
Xp7882      CTGGACCCGACGGCCCTGTTCGATCGTTTGAAGAAGATGCCGGGCCATGACAACTACTAC 657
            ********* ****************************************** ** ***

XccA44      AAGCGCACGGTCGGCGATTTTGCGCGCGATGTGAAGCGCTACGGTCTGTCGGCCACGTTG 720
StmK279a    AAGCGCACGGTCGGCGATTTTGCGCGCGATGTGAAGCGCAACGGCCTGTCGGCCACGTTG 717
Xac1381     AAGCGCACGGTCGGCGATTTTGCACGCGATGTGAAGCGCAACGGTCTGTCGGCCACGTTG 717
Xp7882      AAGCGCACGGTCGGCGATTTCGCGCGCGATGTGAAGCGCAATGGTCTGTCGGCCACGTTG 717
            ******************** ** *************** * ** ***************

XccA44      GAAGATCGCAAGATGTGGGGCGTGATGCGGATGACGCCCACGGATCTGTCCGACGTCAAC 780
StmK279a    GAAGATCGCAAGATGTGGGGCGTGATGCGGATGACGCCCACGGACCTGTCCGACGTCAAC 777
Xac1381     GAGGATCGCAAGATGTGGGGCGTGATGCGGATGACGCCCACGGATCTGTCCGACGTCAAC 777
Xp7882      GAAGATCGCAAGATGTGGGGCGTGATGCGAATGACGCCCACGGATCTGTCCGACGTCAAC 777
            ** ************************** ************** ***************

XccA44      GCCAACACCTACACCTACTTGATGAACGGCACGACCTCACTGGGCAACTGGACCGGTTTG 840
StmK279a    GCCAACACCTACACCTACCTGATGAACGGCACGACCTCACTGGGCAACTGGACCGGTTTG 837
Xac1381     GCCAACACCTACACCTACCTGATGAACGGCACGACCTCTCTGGGCAACTGGACAGGTTTG 837
Xp7882      GCCAACACCTACACCTACCTGATGAACGGCACGACCTCTCTGGGCAACTGGACCGGGTTG 837
            ****************** ******************* ************** ** ***

XccA44      TTCCGCAGTGGCGAGAAGGTGCGCCTGCGTTTCATCAATGGCTCTGCCATGACGTACTTC 900
StmK279a    TTCCGCAGTGGCGAGAAGGTGCGCCTGCGTTTCATCAATGGCTCTGCCATGACGTACTTC 897
Xac1381     TTCCGCAGTGGCGAAAAGGTGCGTCTGCGTTTCATCAATGGCTCTGCCATGACGTACTTC 897
Xp7882      TTCCGCAGTGGCGAGAAGGTGCGTCTGCGTTTCATCAATGGCTCTGCCATGACGTACTTC 897
            ************** ******** ************************************

XccA44      GATGTGCGTATTCCGGGGCTGAAGATGACCGTGGTGGCGGCAGATGGCTTGTATGTCCAT 960
StmK279a    GATGTGCGTATTCCGGGGCTGAAGATGACCGTGGTGGCGGCAGATGGCTTGTATGTCCAT 957
Xac1381     GATGTGCGCATTCCGGGGTTGAAGATGACCGTGGTGGCGGCAGATGGCTTGTACGTCCAT 957
Xp7882      GATGTGCGTATTCCGGGGTTGAAGATGACCGTGGTCGCGGCGGATGGCTTGTACGTCCAT 957
            ******** ********* **************** ***** *********** ******

XccA44      CCGGTTTCCGTCGACGAGTTCCGCATCGCGGTAGCAGAAACCTTCGATGTGATCGTGGAG 1020
StmK279a    CCGGTTTCCGTCGACGAGTTCCGCATCGCGGTAGCAGAAACCTTCGATGTGATCGTGGAG 1017
Xac1381     CCGGTTTCCGTCGACGAGTTCCGCATTGCAGTAGCTGAAACCTTCGATGTGATCGTGGAG 1017
Xp7882      CCGGTTTCGGTCGACGAGTTCCGCATTGCAGTAGCAGAAACCTTCGATGTGATCGTGGAG 1017
            ******** ***************** ** ***** ************************

XccA44      CCCTCCGGGCAGGACGCATTCACCATCTTTGCCCAAGACTCCGGTCGCACCGGCTACATC 1080
StmK279a    CCCTCCGGGCAGGACGCATTCACCATCTTTGCCCAAGACTCCGGTCGCACCGGCTACATC 1077
Xac1381     CCCTCCGGGCAGGACGCATTCACCATCTTTGCCCAGGACTCCGGTCGCACCGGCTACGTC 1077
Xp7882      CCCTCCGGGCAGGACGCATTCACCATCTTTGCCCAGGACTCCGGTCGCACCGGCTACGTC 1077
            ********************************** ********************** **

XccA44      AGCGGCACGCTCGCTGTGCGCGAAGGATTACGCGCGCCCGTTCCGTCTGTGGATCCCCGG 1140
StmK279a    AGCGGCACGCTCGCTGTGCGCGAAGGATTACGCGCGCCCGTTCCGTCTGTGGATCCCCGG 1137
Xac1381     AGCGGCACGCTCGCCGTGCGCGAAGGACTACGCGCGCCTGTTCCGCCTGTGGATCCCCGG 1137
Xp7882      AGCGGCACGCTCGCCGTGCGCGAAGGACTACGCGCGCCTCTTCCGTCTGTGGATCCCCGG 1137
            ************** ************ ********** ***** *************

XccA44      CCGCTGCTGACGATGGCAGACATGGGCATGGATCATGGGTCGATGGATATGTCTGGCGGC 1200
StmK279a    CCGCTGCTGACGATGGCAGACATGGGCATGGATCATGGGTCGATGGATATGTCTGGCGGC 1197
Xac1381     CCGCTGCTGACGATGGCAGACATGGGCATGGATCATGGATCGATGGATATGTCTGGCGGC 1197
Xp7882      CCGCTGCTGACGATGGCAGACATGGGCATGGATCATGGATCGATGGATATGTCTGGCGGC 1197
            ************************************ **********************

XccA44      AGCAAGGGCATGGAAGGCGGCTGTGGTGCGGCCATGGGCATGCCTGGCATGACCCCACCT 1260
StmK279a    AGCAAGGGCATGGAAGGCGGCTGTGGTGCGGCCATGGGCATGCCTGGCATGACCCCACCT 1257
Xac1381     AGCAAGGGCATGGAAGGCGGCTGTGGTGCGGCCATGGGTATGCCCGGCATGGCCCCACCT 1257
Xp7882      AGCAAGGGCATGGAAGGCGGCTGTGGTGCAGCCATGGGCATGCCCGGCATGACCCCACCT 1257
            ***************************** ******** ***** ****** ********

XccA44      GTCAGCGGTAACGCGACCTCGGCCCATGCAGGCCATGCGATGCCCGCCGCCGGCGATGGT 1320
StmK279a    GTCAGCGGTAACGCGACCTCGGCCCATGCAGGCCATGCGATGCCCGCCGCCGGCGATGGT 1317
Xac13       GCCAGCGGTAACGAGACCTCGGCCCATGCAGGCCACGCGATGCCCGCCGCCGGCGATGGT 1317
Xp7882      GCCAGCGGCAATGAGGCCTCGGCCCATGCGGGTCACGCGATGCCCGCCGCCGGCGATGGT 1317
            * ****** ** * * ************* ** ** ************************
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Figure 4-5. Continued

```
XccA44      GCCATGGCAGGCATGCAGCACGGGGGCATGCAATCACACCCTGCTAGCGAGACCAACAAT 1380
StmK279a    GCCATGGCAGGCATGCAGCACGGGGGCATGCAATCACACCCTGCTAGCGAGACCAACAAT 1377
Xac1381     GCCATGGCCGGCATGCAGCACGGGGGCATGCAATCACACCCTGCCAGCGAGACCAACAAT 1377
Xp7882      GCCATGGCCGGCATGCAGCATGGGGGCATGCAATCACACCCTGCCAGCGAGACCAACAAT 1377
            ******** *********** ********************** ***************

XccA44      CCCCTGTTGGACAACCAAGCCATGAGCGTGAGTTCGCGCTTGGATGATCCGGGCAATGGC 1440
StmK279a    CCCCTGTTGGACAACCAAGCCATGAGCGTGAGTTCGCGCTTGGATGATCCGGGCAATGGC 1437
Xac1381     CCCCTGTTGGACAACCAAGCCATGAGCGTGACTTCGCGCTTGGACGATCCGGGCAATGGC 1437
Xp7882      CCCCTGTTGGACAACCAAGCCATGAGCGTGACTTCGCGCTTGGACGATCCGGGCAATGGC 1437
            ****************************** ************ ***************

XccA44      CTGCGCGATAACGGCCGTCATGTGCTGACGTATTCCATGCTCAAGAGCACCTTTGAAGAC 1500
StmK279a    CTGCGCGATAACGGCCGTCATGTGCTGACGTATTCCATGCTCAAGAGCACCTTTGAAGAC 1497
Xac1381     CTGCGCGATAACGGCCGTCATGTACTGACGTATTCCATGCTCAAGAGCACCTTTGAAGAC 1497
Xp7882      CTGCGCGATAACGGCCGTCATGTACTGACGTATTCCATGCTCAAGAGCACCTTTGAAGAC 1497
            ********************** ************************************

XccA44      CCTGACGGACGCGACCCCGGTCGCGAGATCGAGCTGCATCTGACCGGACACATGGAGAAA 1560
StmK279a    CCTGACGGACGCGACCCCGGTCGCGAGATCGAGCTGCATCTGACCGGACACATGGAGAAA 1557
Xac1381     CCTGACGGACGCGACCCCGGTCGCGAGATCGAGCTGCATCTGACCGGACACATGGAGAAA 1557
Xp7882      CCTGACGGACGCGACCCCGGTCGCGAGATCGAGCTGCATCTGACCGGACACATGGAGAAA 1557
            ************************************************************

XccA44      TTCTCCTGGGGCTTCAACGGTCAGAAGTTTTCCGATGTCGAGCCGCTGCGGCTGAACTAC 1620
StmK279a    TTCTCCTGGGGCTTCAACGGTCAGAAGTTTTCCGATGTCGAGCCGCTGCGGCTGAACTAC 1617
Xac1381     TTCTCCTGGGGTTTCAATGGTCAGAAGTTTTCCGATGTCGAGCCGCTGCGGCTGAACTAC 1617
Xp7882      TTCTCCTGGGGTTTCAATGGTCAGAAGTTTTCCGATGTCGAGCCGCTACGGCTGAACTAC 1617
            *********** ***** ***************************** ************

XccA44      GGCGAGCGTATGCGCATCGTATTGGTTAACGACACGATGATGACCCATCCCATCCATTTG 1680
StmK279a    GGCGAGCGTATGCGCATCGTATTGGTTAACGACACGATGATGACCCATCCCATCCATTTG 1677
Xac1381     GGCGAGCGCATGCGCATCGTGTTGGTCAACGACACGATGATGACCCATCCGATCCATTTG 1677
Xp7882      GGCGAGCGCATGCGCATCGTATTGGTCAACGACACGATGATGACCCACCCGATCCATTTG 1677
            ******** *********** ***** ******************** ** *********

XccA44      CACGGCATGTGGAGTGACGTGGAGGACGACAACGGCAACTTCATGGTGCGCAAGCACACG 1740
StmK279a    CACGGCATGTGGAGTGACGTGGAGGACGACAACGGCAACTTCATGGTGCGCAAGCACACG 1737
Xac1381     CATGGCATGTGGAGCGATGTGGAAGACGATAACGGCAACTTCATGGTGCGCAAGCACACG 1737
Xp7882      CACGGCATGTGGAGCGATGTGGAAGACGATAGCGGCAACTTCATGGTGCGCAAGCACACG 1737
            ** *********** ** ***** ***** * ****************************

XccA44      GTGGATATGCCGCCAGGTAGCCGACGCACGTATCGCGTGCGTGCCGATGCGTTGGGCAGC 1800
StmK279a    GTGGATATGCCGCCAGGTAGCCGACGCACGTATCGCGTGCGTGCCGATGCGTTGGGCAGC 1797
Xac1381     GTGGACATGCCGCCGGGTAGCCGACGTACGTATCGCGTGCGTGCCGATGCGTTGGGCAGC 1797
Xp7882      GTGGACATGCCGCCAGGCAGCCGACGTACGTATCGCGTGCGTGCCGATGCGTTGGGCAGC 1797
            ***** ******** ** ******** ********************************

XccA44      TGGGCGTTCCATTGCCACCTGCTTTATCACATGGAAGCCGGAATGATGCGCACGGTGAGG 1860
StmK279a    TGGGCGTTCCATTGCCACCTGCTTTATCACATGGAAGCCGGAATGATGCGCACGGTGAGG 1857
Xac1381     TGGGCGTTCCATTGCCACCTGCTTTATCACATGGAAGCCGGAATGATGCGCACGGTGAGG 1857
Xp7882      TGGGCGTTCCATTGCCACCTGCTCTATCACATGGAAGCCGGAATGATGCGCACGGTGAGG 1857
            ********************** ************************************

XccA44      GTCGACGAATGA 1872
StmK279a    GTCGACGAATGA 1869
Xac1381     GTCGACGAATGA 1869
Xp7882      GTCGACGAATGA 1869
            ************
```

Figure 4-5. Continued

```
XccA44       MSHDDFRGPHGGPPLLPSRRRFVQGLALGGAVAGLGFWPKASWALKGPGQPNVLSGTEFD 60
StmK279a     MSHDDFRGPRGGP-LLPSRRRFVQGLALGGAVAGLGFWPKASWALKGPGQPNVLSGTEFD 59
Xac1381      MSHDDFRGPHGGP-LLPSRRRFVQGLALGGAVAGLGFWPKASWALKGPGQANVLSGTEFD 59
Xp7882       MSHDDFRGPHGGP-LLPSRRRFVQGLALGGAVAGLGFWPKASWALKGPGQANVLSGTEFD 59
             *********:*** ***********************************.********* 
XccA44       LTIGETPMNFTGKTRTAITVNGSVPAPLLRWREGTTVSLRVSNALPANSLHGTDTSIHWH 120
StmK279a     LTIGETPMNFTGKTRTAITVNGSVPAPLLRWREGTTVNLRVSNALPANSIHGADTSIHWH 119
Xac1381      LTIGETPMNFTGKTRTAITVNGSVPAPLLRWREGTTVNLRVSNALPANSLHGTDTSIHWH 119
Xp7882       LTIGETPMNFTGKTRTAITVNGSVPAPLLRWREGTTVNLRVSNALPANSLHGTDTSIHWH 119
             ************************************.***********:**:******* 
XccA44       GIILPANMDGVPGLSFDGIGRGETYHYRFTLHQGGTYWYHSHSGFQEQAGLYGPIVIDPL 180
StmK279a     GIILPANMDGVPGLSFDGIGRGETYHYRFTLHQGGTYWYHSHSGFQEQAGLYGPIVIDPL 179
Xac1381      GIILPANMDGVPGLSFDGIGRGETYHYRFTLHQGGTYWYHSHSGFQEQAGLYGPIVIDPL 179
Xp7882       GIILPANMDGVPGLSFDGIGRGETYHYRFTLHQGGTYWYHSHSGFQEQAGLYGPIVIDPL 179
             ************************************************************ 
XccA44       EPEPFSFDRDYVVMLSDWTDLDPTALFDRLKKMPGHDNYYKRTVGDFARDVKRYGLSATL 240
StmK279a     EPEPFSFDRDYVVMLSDWTDLDPTALFDRLKKMPGHDNYYKRTVGDFARDVKRNGLSATL 239
Xac1381      EPEPFSFDRDYVVMLSDWTDLDPAALFDRLKKMPGHDNYYKRTVGDFARDVKRNGLSATL 239
Xp7882       EPEPFSFDRDYVVMLSDWTDLDPTALFDRLKKMPGHDNYYKRTVGDFARDVKRNGLSATL 239
             ***********************:**************************** ****** 
XccA44       EDRKMWGVMRMTPTDLSDVNANTYTYLMNGTTSLGNWTGLFRSGEKVRLRFINGSAMTYF 300
StmK279a     EDRKMWGVMRMTPTDLSDVNANTYTYLMNGTTSLGNWTGLFRSGEKVRLRFINGSAMTYF 299
Xac1381      EDRKMWGVMRMTPTDLSDVNANTYTYLMNGTTSLGNWTGLFRSGEKVRLRFINGSAMTYF 299
Xp7882       EDRKMWGVMRMTPTDLSDVNANTYTYLMNGTTSLGNWTGLFRSGEKVRLRFINGSAMTYF 299
             ************************************************************ 
XccA44       DVRIPGLKMTVVAADGLYVHPVSVDEFRIAVAETFDVIVEPSGQDAFTIFAQDSGRTGYI 360
StmK279a     DVRIPGLKMTVVAADGLYVHPVSVDEFRIAVAETFDVIVEPSGQDAFTIFAQDSGRTGYI 359
Xac1381      DVRIPGLKMTVVAADGLYVHPVSVDEFRIAVAETFDVIVEPSGQDAFTIFAQDSGRTGYV 359
Xp7882       DVRIPGLKMTVVAADGLYVHPVSVDEFRIAVAETFDVIVEPSGQDAFTIFAQDSGRTGYV 359
             ************************************************************: 
XccA44       SGTLAVREGLRAPVPSVDPRPLLTMADMGMDHGSMDMSGGSKGMEGGCGAAMGMPGMTPP 420
StmK279a     SGTLAVREGLRAPVPSVDPRPLLTMADMGMDHGSMDMSGGSKGMEGGCGAAMGMPGMTPP 419
Xac1381      SGTLAVREGLRAPVPPVDPRPLLTMADMGMDHGSMDMSGGSKGMEGGCGAAMGMPGMAPP 419
Xp7882       SGTLAVREGLRAPLPSVDPRPLLTMADMGMDHGSMDMSGGSKGMEGGCGAAMGMPGMTPP 419
             *************:*.****************************************:** 
XccA44       VSGNATSAHAGHAMPAAGDGAMAGMQHGGMQSHPASETNNPLLDNQAMSVSSRLDDPGNG 
StmK279a     VSGNATSAHAGHAMPAAGDGAMAGMQHGGMQSHPASETNNPLLDNQAMSVSSRLDDPGNG 479
Xac1381      ASGNETSAHAGHAMPAAGDGAMAGMQHGGMQSHPASETNNPLLDNQAMSVTSRLDDPGNG 479
Xp7882       ASGNEASAHAGHAMPAAGDGAMAGMQHGGMQSHPASETNNPLLDNQAMSVTSRLDDPGNG 479
             .*** :****************************************:********* 
XccA44       LRDNGRHVLTYSMLKSTFEDPDGRDPGREIELHLTGHMEKFSWGFNGQKFSDVEPLRLNY 540
StmK279a     LRDNGRHVLTYSMLKSTFEDPDGRDPGREIELHLTGHMEKFSWGFNGQKFSDVEPLRLNY 539
Xac1381      LRDNGRHVLTYSMLKSTFEDPDGRDPGREIELHLTGHMEKFSWGFNGQKFSDVEPLRLNY 539
Xp7882       LRDNGRHVLTYSMLKSTFEDPDGRDPGREIELHLTGHMEKFSWGFNGQKFSDVEPLRLNY 539
             ************************************************************ 
XccA44       GERMRIVLVNDTMMTHPIHLHGMWSDVEDDNGNFMVRKHTVDMPPGSRRTYRVRADALGS 600
StmK279a     GERMRIVLVNDTMMTHPIHLHGMWSDVEDDNGNFMVRKHTVDMPPGSRRTYRVRADALGS 599
Xac1381      GERMRIVLVNDTMMTHPIHLHGMWSDVEDDNGNFMVRKHTVDMPPGSRRTYRVRADALGS 599
Xp7882       GERMRIVLVNDTMMTHPIHLHGMWSDVEDDSGNFMVRKHTVDMPPGSRRTYRVRADALGS 599
             ****************************.*********************************** 
XccA44       WAFHCHLLYHMEAGMMRTVRVDE- 623
StmK279a     WAFHCHLLYHMEAGMMRTVRVDE- 622
Xac1381      WAFHCHLLYHMEAGMMRTVRVDE- 622
Xp7882       WAFHCHLLYHMEAGMMRTVRVDE- 622
             ********************** 
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Figure 4-6. Alignment of complete amino acid sequences of *copA.* XccA44, *Xanthomonas citri* subsp. *citri* A44; StmK279a, *Stenotrophomonas maltophilia* K279a; Xac1381, *X. alfalfae* subsp. *citrumelonis* 1381; Xp7882, *X. perforans* 7882. The homology line indicates the degree of amino acid homology among all aligned sequence members. Asterisk (*), identical residue; colon (**:**), conserved substitution; period (**.**), semiconserved substitution; gap, no conservation (Thompson et al., 1994).

```
XccA44      ATGAACATCAATAGACGCGATACCACCCTGACTGCGCTGAC---GGCTATCTCGCTGGCC 57
StmK279a    ATGAACATCAATAGACGCGATACCACCCTGACTGCGCTGAC---GGCTATCTCGCTGGCC 57
Xac1381     ATGAACATCAATAGACGCGATACCACCCTGACTGCGCTGACGCTGGCTATCTCGCTGGCC 60
Xp7882      ATGAACATCAATAGACGCAATACCACCCTGACTGCGCTGACCCTGGCTATCTCGCTGGCC 60
            ****************** ********************   ***************

XccA44      CTGGCCAATGCGGCCAGCGCCCAATCTATGCAGCACGGCTCCATGCAGATGGAGCAGGGC 117
StmK279a    CTGGCCAATGCGGCCAGCGCCCAATCTATGCAGCACGGCTCCATGCAGATGGAGCAGGGC 117
Xac1381     CTGGCCAGTGCGGCCAGCGCCCAATCCATGCAGCACGGCTCCATGCCGATGGGGCAGAGC 120
Xp7882      CTGGCCAATGCGGCCAGCGCCCAATCCATGCAGCACGGCTCCATGCCGATGGAGCAGGGC 120
            ******* ****************** ******************* ***** **** **

XccA44      GCGCAGACCCAGACTCAGGATCACTCTGCACATCAGGCGCCGACATCAAAACCTGCGCCA 177
StmK279a    GCGCAGACCCAGACTCAGGATCACTCTGCACATCAGGCGCCGACATCAAAACCTGCGCCA 177
Xac1381     GCGCAGACCCAGACGCAGGATCACTCTGCACATCAGGCGCCGACATCAAAACCTGCGCCC 180
Xp7882      GCGCAGACCCAGGCTCAGGATCACTCTGCACATCAGGCGCCGACATCAAAGCCTGCGCCA 180
            ************ * ********************************** ********

XccA44      GCCCAAAAGCCTGCAACACCGGCCAAGACGAGCGAAGCGACGATCGATCATGCGGCGATG 237
StmK279a    GCCCAAAAGCCTGCAACACCGGCCAAGACGAGCGAAGCGACGATCGATCATGCGGCGATG 237
Xac1381     ACCCAAAAGCCTGCAACACCGGCCAAGACCAGCGAAGCGACCATCGATCATGCGGCGATG 240
Xp7882      GCCCCCAAACCTACAACACCGGCCAAGACCAGCGAAGCGACTATCGATCATGCGGCGATG 240
             ***  ** *** ***************** ************ ******************

XccA44      GGCCACGCCGAGCCGCAGGCTAACGCAGCCGAGCCTGCCATGCAGGGCATGGACCATTCG 297
StmK279a    GGCCACGCCGAGCCGCAGGCTAACGCAGCCGAGCCTGCCATGCAGGGCATGGACCATTCG 297
Xac1381     GGTCATCCCGCGCCACCGGCTAAAGCAGCCGAGCCTGCGATGCAAGGGATGGACCATTCC 300
Xp7882      GGCCATGCCGCGCCGCCGGCTCAAGCAGCCGAGCCTGCCATGCAGGGCATGGACCACTCG 300
            ** **  *** *** * **** * ************** ***** ** ******** **

XccA44      CAGATGGGGCACGGCTCGCCCGCGAGCACACCTGCGGCGCCCACAGCGCAGGCGCAGTCG 357
StmK279a    CAGATGGGGCACGGCTCGCCCGCGAGCACACCTGCGGCGCCCACAGCGCAGGCGCAGTCG 357
Xac1381     AAGATGGGGCACGGCTCGCCTCCAAGTACGCCTGTAGCGCCCAAAGCGCAGACGCAGTCG 360
Xp7882      CAGATGGGGCACAGCTCGCCCGCGAGTACACCTGCAGCGCCCAAAGCGCAGACGCAGCCG 360
             *********** *******  * ** ** ****  ******* ******* ***** **

XccA44      ATGCAGGGCATGGATCACAGTCAGATGGCACAGCCCGCTGCAGCCGACACCTCCGGCACG 417
StmK279a    ATGCAGGGCATGGATCACAGTCAGATGGCACAGCCCGCTGCAGCCGACACCTCCGGCACG 417
Xac1381     ATGCAGGGCATGGATCACAGTCAGATGGCACAGCCCGCTGCAGCCGACACCTCCA---TG 417
Xp7882      ATGCAGGGCATGGATCACAGTCAGATGGCACAGCCCGCTGCGTCCGACGCCTCCAGCACG 420
            ************************************  ***** *****     *

XccA44      GCCACGCCTGCGATGCAGGGGATGGACCATTCGCAGATGGGCCATGATTCGCCCGCGCCC 477
StmK279a    GCCACGCCTGCGATGCAGGGGATGGACCATTCGCAGATGGGCCATGATTCGCCCGCGCCC 477
Xac1381     ACCACACCTGCGATGCAGGGGATGGACCATTCGCAGATGGGGCATGGTTCGCCCGCACCC 477
Xp7882      TCCACGCCTGCGATGCAGGGGATGGACCATTCGCAGATGGGCCACGGTTCGCCCGCATCC 480
             **** ********************************** ** * *********  **

XccA44      GCTACACCAGAAGCGGGAATGCAATCGATGGAGGGCATGGACCACAGTCAGATGGGACAC 537
StmK279a    GCTACACCAGAAGCGGGAATGCAATCGATGGAGGGCATGGACCACAGTCAGATGGGACAC 537
Xac1381     GCTACGCCAGAAGCGGGTATGCAATCAATGGAAGGCATGGACCACAGTCAGATGGGACAC 537
Xp7882      GCTACGCCAGAAGCTGGCATGCAATCGATGGAGGGCATGGACCACAGTCAGATGGGACAT 540
            ***** ******** ** ******** ***** *************************

XccA44      GGGCCTGCAGCGCCAACGCAGCCGCGCACCCCGATTCCCGCGGTGACGGACGCGGATCGC 597
StmK279a    GGGCCTGCAGCGCCAACGCAGCCGCGCACCCCGATTCCCGCGGTGACGGACGCGGATCGC 597
Xac1381     GGGTCTGCAGCGCCAACGCAGCCGCGCACCCCGATTCCGGCGGTGACCGAGGCTGATCGT 597
Xp7882      GGGCCTGTCGCACCAACGCAGCCGCGCACCCCGATTCCTGCGGTGACCGAGGCTGATCGT 600
            *** ***  ** ************************* ******** ** ** *****

XccA44      AAGGCGGCCATCGCGCCGGAACACGCGCATCCGGTGCATGACAACTCGATTAAGAGCTAC 657
StmK279a    AAGGCGGCCATCGCGCCGGAACACGCGCATCCGGTGCATGACAACTCGATCAAGAGCTAC 657
Xac1381     CAGGCGGCCATCGCGCCCGCACACGCGCATCCGGTGCATGACAATTCGATCAAGAGCTAT 657
Xp7882      CAGGCGGCCATCGCGCCCGCACACGCGCATCCGGTGCATGACAATTCGATCAAGAGCTAC 660
             ***************** * ************************ ***** ********
```

Figure 4-7. Alignment of complete nucleotide sequences of *copB.* XccA44, *Xanthomonas citri* subsp. *citri* A44; StmK279a, *Stenotrophomonas maltophilia* K279a; Xac1381, *X. alfalfae* subsp. *citrumelonis* 1381; Xp7882, *X. perforans* 7882. The homology line indicates the degree of amino acid homology among all aligned sequence members. Asterisk (*), identical residue; gap, no conservation (Thompson et al., 1994).

```
XccA44      GTGCTGCTCAATCGCCTGGAAACCTGGGATGCCGATCCGGGCACCGGGCTGGGCTGGGAG 717
StmK279a    GTGCTGCTCAATCGCCTGGAAACCTGGGATGCCGATCCGGGCACCGGGCTGGGCTGGGAG 717
Xac1381     GTACTGCTCAATCGCCTGGAAGCCTGGGATGCCGATCCTGGAACCGGGCTGGGTTGGGAG 717
Xp7882      GTACTGCTCAATCGCCTGGAAGCCTGGGATGCCGATCCGGGCACCGGGCTGGGTTGGGAG 720
            ** ****************** ***************** ** *********** ******

XccA44      GGCCAGGGCTGGATCGGTACGGACCTCAATCGCGTCTGGCTCCGCAGTGAAGGCGAACGC 777
StmK279a    GGCCAGGGCTGGATCGGTACGGACCTCAATCGCGTCTGGCTCCGCAGTGAAGGCGAACGC 777
Xac1381     GGTCAGGGCTGGATCGGTACGGACCTCAATCGCGTCTGGTTGCGCAGTGAAGGCGAACGC 777
Xp7882      GGTCAGGGTTGGATCGGTACGGACCTCAATCGCGTCTGGTTCCGCAGTGAAGGCGAACGC 780
            ** ***** ****************************** * ******************

XccA44      ACGGATGGTCAGACCGAGTCGGCTGATCTGGAAGTGCTTTACGGCCGCAGTATCTCCACG 837
StmK279a    ACGGATGGTCAGACCGAGTCGGCTGATCTGGAAGTGCTTTACGGCCGCAGTATCTCCACG 837
Xac1381     ACAGATGGTCAGACAGAATCGGCTGATCTGGAAGTGCTCTACGGCCGCAGTATCTCCACG 837
Xp7882      ACAGATGGTCAGACCGAGTCGGCTGATCTGGAAGTGCTCTACGGCCGCAGTATCTCCACG 840
            ** *********** ** ******************** **********************

XccA44      TGGTGGGATGTGGTGGCCGGTGTGCGTCATGACTTCAAGCCTGGGGCATCGCAGAACTTC 897
StmK279a    TGGTGGGATGTGGTGGCCGGTGTGCGTCATGACTTCAAGCCTGGGGCATCGCAGAACTTC 897
Xac1381     TGGTGGGATGTGGTGGCCGGTGTGCGCCATGACTTCAAGCCGGGGGCTTCGCAGAACTTC 897
Xp7882      TGGTGGGATGTGGTAGCCGGTGTGCGCCATGACTTCAAGCCGGGGGCTTCGCAGAACTTC 900
            ************** *********** ************** ***** ************

XccA44      GCCGCTATCGGTGTACAGGGCTTGGCGCCGATGAAGTTCGAAGTGTCCGCCACAGCCTAT 957
StmK279a    GCCGCTATCGGTGTACAGGGCTTGGCGCCGATGAAGTTCGAAGTGTCCGCCACAGCCTAT 957
Xac1381     GCCGCTATCGGCGTACAGGGCTTGGCACCGATGAAGTTCGAAGTGTCCGCCACAGCCTAT 957
Xp7882      GCCGCTATCGGCGTACAGGGCTTGGCACCGATGAAGTTCGAAGTATCCGCCACAGCCTAT 960
            *********** ************** ***************** ************

XccA44      CTCGGCGAAGGGGGCCAGACTGCCGCCAATGTCGAGGCCGAGTACGAATTGCTGCTAACC 1017
StmK279a    CTCGGCGAAGGGGGCCAGACTGCCGCCAATGTCGAGGCCGAGTACGAGTTGCTGCTAACC 1017
Xac1381     CTCGGCGAAGGCGGCCAGACTGCCGCCAATGTCGAGGCTGAGTACGAACTGCTGCTGACC 1017
Xp7882      CTCGGCGAAGGCGGCCAGACTGCTGCCAATGTCGAGGCCGAGTACGAACTGCTGCTGACC 1020
            *********** *********** ************** ********  *******

XccA44      AACCGGCTGATCTTGCAGCCGCTGGTGGAAGTCACCGCCTATGGCAAGAACGATCCATTG 1077
StmK279a    AACCGGCTGATCTTGCAGCCGCTGGTGGAAGTCACCGCCTATGGCAAGAACGATCCATTG 1077
Xac1381     AATCGGCTGATCCTACAGCCGCTGGTGGAGGTCACGGCGTATGGCAAGAACGATCCACTG 1077
Xp7882      AATCGGCTGATCCTGCAGCCGCTGGTGGAGGTCACGGCGTATGGCAAGAACGATCCATTG 1080
            ** ********* * ************** ***** ** ******************

XccA44      CGCGGGATAGGTTCGGGTCTGAGTGCCGCTGAGGCGGGGCTACGACTTCGCTATGAGTTC 1137
StmK279a    CGCGGGATAGGTTCGGGTCTGAGTGCCGCTGAGGCGGGGCTACGACTTCGCTATGAGTTC 1137
Xac1381     CGCGGAATAGGTTCGGGTCTGAGTACCGCTGAGGCGGGCCTGCGACTTCGTTATGAGTTC 1137
Xp7882      CGCGGAATAGGTTCGGGTCTGAGTACCGCTGAGGCGGGCCTGCGACTTCGTTATGAGTTC 1140
            ***** ****************** ************* ** ******** ******

XccA44      ACCCGAAAGTTCGCTCCCTACATCGGCGTGGTGTACGAGCGCGCGTTTGGCAATACCGCA 1197
StmK279a    ACCCGAAAGTTCGCTCCCTACATCGGCGTGGTGTACGAGCGCGCGTTTGGCAATACCGCA 1197
Xac1381     ACCCGAAAGTTCGCTCCCTACATCGGCGTGGTGTACGAGCGTGCGTTTGGCAATACCGCA 1197
Xp7882      ACCCGAAAGTTCGCTCCCTACATCGGCGTGGTGTACGAGCGTGCGTTTGGCAATACCGCA 1200
            ************************************** ***************

XccA44      GACATGCGACGCGAGCATGGCGAGTCCTTTGAAGACACGCGCTTGGTCATCGGCCTTCGT 1257
StmK279a    GACATGCGACGCGAGCATGGCGAGTCCTTTGAAGACACGCGCTTGGTCATCGGCCTTCGT 1257
Xac1381     GACATGCGACGCGAGCATGGCGAGTCCTTTGAAGACACGCGCTTGGTCGTCGGCCTTCGT 1257
Xp7882      GACATGCGACGCGAGCATGGCGAGTCTTTTGAAGACACGCGCTTGGTCATCGGCCTTCGT 1260
            ************************* ********************* ********

XccA44      ACCTGGTTCTAA 1269
StmK279a    ACCTGGTTCTAA 1269
Xac1381     ACCTGGTTCTAA 1269
Xp7882      ACCTGGTTCTAA 1272
            ************
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Figure 4-7. Continued

```
XccA44       MNINRRDTTLTALT-AISLALANAASAQSMQHGSMQMEQGAQTQTQDHSAHQAPTSKPAP 59
StmK279a     MNINRRDTTLTALT-AISLALANAASAQSMQHGSMQMEQGAQTQTQDHSAHQAPTSKPAP 59
Xcc1381      MNINRRDTTLTALTLAISLALASAASAQSMQHGSMPMGQSAQTQTQDHSAHQAPTSKPAP 60
Xp7882       MNINRRNTTLTALTLAISLALANAASAQSMQHGSMPMEQGAQTQAQDHSAHQAPTSKPAP 60
             ******:******* *******.*********** * *.****:***************

XccA44       AQKPATPAKTSEATIDHAAMGHAEPQANAAEPAMQGMDHSQMGHGSPASTPAAPTAQAQS 119
StmK279a     AQKPATPAKTSEATIDHAAMGHAEPQANAAEPAMQGMDHSQMGHGSPASTPAAPTAQAQS 119
Xcc1381      TQKPATPAKTSEATIDHAAMGHPAPPAKAAEPAMQGMDHSKMGHGSPPSTPVAPKAQTQS 120
Xp7882       APKPTTPAKTSEATIDHAAMGHAAPPAQAAEPAMQGMDHSQMGHSSPASTPAAPKAQTQP 120
             : **:*****************. * *:************:***.**.***.**.**:*.

XccA44       MQGMDHSQMAQPAAADTSGTATPAMQGMDHSQMGHDSPAPATPEAGMQSMEGMDHSQMGH 179
StmK279a     MQGMDHSQMAQPAAADTSGTATPAMQGMDHSQMGHDSPAPATPEAGMQSMEGMDHSQMGH 179
Xcc1381      MQGMDHSQMAQPAAADTSMT-TPAMQGMDHSQMGHGSPAPATPEAGMQSMEGMDHSQMGH 179
Xp7882       MQGMDHSQMAQPAASDASSTSTPAMQGMDHSQMGHGSPASATPEAGMQSMEGMDHSQMGH 180
             **************:*:* * **************.***.********************

XccA44       GPAAPTQPRTPIPAVTDADRKAAIAPEHAHPVHDNSIKSYVLLNRLETWDADPGTGLGWE 239
StmK279a     GPAAPTQPRTPIPAVTDADRKAAIAPEHAHPVHDNSIKSYVLLNRLETWDADPGTGLGWE 239
Xcc1381      GSAAPTQPRTPIPAVTEADRQAAIAPAHAHPVHDNSIKSYVLLNRLEAWDADPGTGLGWE 239
Xp7882       GPVAPTQPRTPIPAVTEADRQAAIAPAHAHPVHDNSIKSYVLLNRLEAWDADPGTGLGWE 240
             *..*************:***:***** ********************:************

XccA44       GQGWIGTDLNRVWLRSEGERTDGQTESADLEVLYGRSISTWWDVVAGVRHDFKPGASQNF 299
StmK279a     GQGWIGTDLNRVWLRSEGERTDGQTESADLEVLYGRSISTWWDVVAGVRHDFKPGASQNF 299
Xcc1381      GQGWIGTDLNRVWLRSEGERTDGQTESADLEVLYGRSISTWWDVVAGVRHDFKPGASQNF 299
Xp7882       GQGWIGTDLNRVWFRSEGERTDGQTESADLEVLYGRSISTWWDVVAGVRHDFKPGASQNF 300
             *************:********************************************

XccA44       AAIGVQGLAPMKFEVSATAYLGEGGQTAANVEAEYELLLTNRLILQPLVEVTAYGKNDPL 359
StmK279a     AAIGVQGLAPMKFEVSATAYLGEGGQTAANVEAEYELLLTNRLILQPLVEVTAYGKNDPL 359
Xcc1381      AAIGVQGLAPMKFEVSATAYLGEGGQTAANVEAEYELLLTNRLILQPLVEVTAYGKNDPL 359
Xp7882       AAIGVQGLAPMKFEVSATAYLGEGGQTAANVEAEYELLLTNRLILQPLVEVTAYGKNDPL 360
             ************************************************************

XccA44       RGIGSGLSAAEAGLRLRYEFTRKFAPYIGVVYERAFGNTADMRREHGESFEDTRLVIGLR 419
StmK279a     RGIGSGLSAAEAGLRLRYEFTRKFAPYIGVVYERAFGNTADMRREHGESFEDTRLVIGLR 419
Xcc1381      RGIGSGLSTAEAGLRLRYEFTRKFAPYIGVVYERAFGNTADMRREHGESFEDTRLVVGLR 419
Xp7882       RGIGSGLSTAEAGLRLRYEFTRKFAPYIGVVYERAFGNTADMRREHGESFEDTRLVIGLR 420
             ********:*************************************************:***

XccA44       TWF- 422
StmK279a     TWF- 422
Xcc1381      TWF- 422
Xp7882       TWF- 423
             ***
```

Figure 4-8. Alignment of complete amino acid sequences of *copB.* XccA44, *Xanthomonas citri* subsp. *citri* A44; StmK279a, *Stenotrophomonas maltophilia* K279a; Xac1381, *X. alfalfae* subsp. *citrumelonis* 1381; Xp7882, *X. perforans* 7882. The homology line indicates the degree of amino acid homology among all aligned sequence members. Asterisk (*), identical residue; colon (**:**), conserved substitution; period (**.**), semiconserved substitution; gap, no conservation (Thompson et al., 1994).

```
XccA44      ATGAACATCAATAGACGCGATACCACCCTGACTGCGCTGACG---GCTATCTCGCTGGCC 57
Xsp1219     ATGAACATCAATAGACGCGATACCACCCTGACTGCGCTGACGCTGGCTATCTCGCTGGCC 60
Xe81-23     ATGAACATCAATAGACGCGATACCACCCTGACTGCGCTGACG---GCTATCTCGCTGGCC 57
            ************************************   ***************

XccA44      CTGGCCAATGCGGCCAGCGCCCAATCTATGCAGCACGGCTCCATGCAGATGGAGCAGGGC 117
Xsp1219     CTGGCCAATGCCGCCAGCGCCCAATCCATGCAGCACGGCTCCATGCCGATGGAGCAGGGC 120
Xe81-23     CTGGCCAATGCGGCCAGCGCCCAATCTATGCAGCACGGCTCCATGCAGATGGAGCAGGGC 117
            *********** ************** ******************* *************

XccA44      GCGCAGACCCAGACTCAGGATCACTCTGCACATCAGGCGCCGACATCAAAACCTGCGCCA 177
Xsp1219     GCGCAGACCCAGACTCAGGATCACTCTGCACATCAGGCGCCGACATCAAAACCTGCGCCA 180
Xe81-23     GCGCAGACCCAGACTCAGGATCACTCTGCACATCAGGCGCCGACATCAAAACCTGCGCCA 177
            ************************************************************

XccA44      GCCCAAAAGCCTGCAACACCGGCCAAGACGAGCGAAGCGACGATCGATCATGCGGCGATG 237
Xsp1219     GCCAAAAAGCCTGCAACACCGGCCAAGAAGAGCGAAGCGACGATCGATCATGCGGCGATG 240
Xe81-23     GCCCAAAAGCCTGCAACACCGGCCAAGACGAGCGAAGCGACGATCGATCATGCGGCGATG 237
            *** ********************** ******************************

XccA44      GGCCACGCCGAGCCGCAGGCTAACGCAGCCGAGCCTGCCATGCAGGGCATGGACCATTCG 297
Xsp1219     GGCCACGCCGCGCCGCCGGCTAAAGCAGCCGAGCCTGCCATGCAGGGCATGGACCATTCG 300
Xe81-23     GGCCACGCCGAGCCGCAGGCTAACGCAGCCGAGCCTGCCATGCAGGGCATGGACCATTCG 297
            ********** ***** ****** ***********************************

XccA44      CAGATGGGGCACGGCTCGCCCGCGAGCACACCTGCGGCGCCCACAGCGCAGGCGCAGTCG 357
Xsp1219     CAGATGGGGCACGGCTCGCCCGCGAGCACACCTGCGGCGCCCACAGCACAGGCGCAGTCG 360
Xe81-23     CAGATGGGGCACGGCTCGCCCGCGAGCACACCTGCGGCGCCCACAGCGCAGGCGCAGTCG 357
            ********************************************* ************

XccA44      ATGCAGGGCATGGATCACAGTCAGATGGCACAGCCCGCTGCAGCCGACACCTC------- 410
Xsp1219     ATGCAGGGCATGGATCACAGTCAGATGGCACAGCCCGCTGCAGCCGACACCTCTGGTACG 420
Xe81-23     ATGCAGGGCATGGA---------------------------------------------- 371
            **************

XccA44      ------------------------------------------------------------
Xsp1219     GCCACGCCTGCGATGCAGGGGATGGACCATTCCAAGATGGGGCACGGCGCGCCTCCGAGC 480
Xe81-23     ------------------------------------------------------------

XccA44      ------------------------------------------------------------
Xsp1219     ACGCCCGCAACGCCCAAAGCGCAGACACAGTCGATGCAGGGCATGGATCACAGTCAGATG 540
Xe81-23     ------------------------------------------------------------

XccA44      -------------------------CGGCACGGCCACGCCTGCGATGCAGGGGATGGAC 444
Xsp1219     GCACAGCCCGCGGCGGCCGACACCTCCGGCACGGCCACGCCTGCGATGCAGGGGATGGAC 600
Xe81-23     ------------------------------------------------------------

XccA44      CATTCGCAGATGGGCCATGATTCGCCCGCGCCCGCTACACCAGAAGCGGGAATGCAATCG 504
Xsp1219     CATTCGCAGATGGGCCATGATTCGCCCGCGTCCGCTACACCAGAAGCGGGAATGCAATCG 660
Xe81-23     ------------------------------------------------------------

XccA44      ATGGAGGGCATGGACCACAGTCAGATGGGACACGGGCCTGCAGCGCCAACGCAGCCGCGC 564
Xsp1219     ATGGAGGGCATGGACCACAGTCAGATGGGACACGGGCCTGCAGCGCCAACGCAGCCGCGC 720
Xe81-23     --------------CCACAGTCAGATGGGACACGGGCCTGCAGCGCCAACGCAGCCGCGC 417
                          ******************************************

XccA44      ACCCCGATTCCCGCGGTGACGGACGCGGATCGCAAGGCGGCCATCGCGCCGGAACACGCG 624
Xsp1219     ACCCCGATACCCGCGGTGACGGACGCGGATCGCAAGGCGGCCATCGCGCCGGAACACGCG 780
Xe81-23     ACCCCGATTCCCGCGGTGACGGACGCGGATCGCAAGGCGGCCATCGCGCCGGAACACGCG 477
            ******** **************************************************

XccA44      CATCCGGTGCATGACAACTCGATTAAGAGCTACGTGCTGCTCAATCGCCTGGAAACCTGG 684
Xsp1219     CATCCGGTGCATGACAACTCGATCAAGAGCTATGTGCTGCTCAATCGCCTGGAAACCTGG 840
Xe81-23     CATCCGGTGCATGACAACTCGATCAAGAGCTACGTGCTGCTCAATCGCCTGGAAACCTGG 537
            ********************** ******** *************************
```

Figure 4-9. Alignment of complete amino acid sequences of *copB* from *Xanthomonas citri* subsp. *citri* A44 (XccA44), *Xanthomonas* sp. (Xsp1219), and *Xanthomonas euvesicatoria* 81-23 (Xe81-23). Asterisk (*), identical residue; gap, no conservation (Thompson et al., 1994).

```
XccA44      GATGCCGATCCGGGCACCGGGCTGGGCTGGGAGGGCCAGGGCTGGATCGGTACGGACCTC 744
Xsp1219     GATGCCGATCCGGGCACCGGGTTTGGCTGGGAGGGCCAAGGCTGGATCGGTACGGACCTC 900
Xe81-23     GATGCCGATCCGGGCACCGGGCTGGGCTGGGAGGGCCAGGGCTGGATCGGTACGGACCTC 597
            ********************* * ************** ********************** 
XccA44      AATCGCGTCTGGCTCCGCAGTGAAGGCGAACGCACGGATGGTCAGACCGAGTCGGCTGAT 804
Xsp1219     AATCGCGTCTGGCTCCGCAGTGAAGGCGAACGCACGGATGGTCGGACCGAGTCGGCTGAT 960
Xe81-23     AATCGCGTCTGGCTCCGCAGTGAAGGCGAACGCACGGATGGTCAGACCGAGTCGGCTGAT 657
             **************************************** ***************** 
XccA44      CTGGAAGTGCTTTACGGCCGCAGTATCTCCACGTGGTGGGATGTGGTGGCCGGTGTGCGT 864
Xsp1219     CTGGAAGTGCTTTACGGCCGCAGTATCTCCACGTGGTGGGATGTGGTGGCCGGTGTGCGT 1020
Xe81-23     CTGGAAGTGCTTTACGGCCGCAGTATCTCCACGTGGTGGGATGTGGTGGCCGGTGTGCGT 717
            ************************************************************ 
XccA44      CATGACTTCAAGCCTGGGGCATCGCAGAACTTCGCCGCTATCGGTGTACAGGGCTTGGCG 924
Xsp1219     CATGACTTCAAGCCTGGGGCATCGCAGAACTTCGCCGCTATCGGTGTACAGGGCTTGGCG 1080
Xe81-23     CATGACTTCAAGCCTGGGGCATCGCAGAACTTCGCCGCTATCGGTGTACAGGGCTTGGCG 777
            ************************************************************ 
XccA44      CCGATGAAGTTCGAAGTGTCCGCCACAGCCTATCTCGGCGAAGGGGGCCA---------- 974
Xsp1219     CCGATGAAGTTCGAAGTGTCCGCCACAGCCTATCTCGGCGAAGGGGGCCA---------- 1130
Xe81-23     CCGATGAAGTTCGAAGTGTCCGCCACAGCCTATCTCGGCGAAGGGGGCCAAGTGTCCGCC 837
            ********************************************** 
XccA44      --------------------------GACTGCCGCCAATGTCGAGGCCGAGTACGAATTG 1008
Xsp1219     --------------------------GACGGCCGCCAATGTCGAGGCCGAGTACGAATTG 1164
Xe81-23     ACAGCCTATCTCGGCGAAGGGGGCCAGACTGCCGCCAATGTCGAGGCCGAGTACGAATTG 897
                                     *** ****************************** 
XccA44      CTGCTAACCAACCGGCTGATCTTGCAGCCGCTGGTGGAAGTCACCGCCTATGGCAAGAAC 1068
Xsp1219     CTGCTAACCAACCGGCTGATCTTGCAGCCGCTGGTGGAAGTCACCGCCTATGGCAAGAAC 1224
Xe81-23     CTGCTAACCAACCGGCTGATCTTGCAGCCGCTGGTGGAAGTCACCGCCTATGGCAAGAAC 957
            ************************************************************ 
XccA44      GATCCATTGCGCGGGATAGGTTCGGGTCTGAGTGCCGCTGAGGCGGGGCTACGACTTCGC 1128
Xsp1219     GATCCATTGCGCGGGATAGGTTCGGGTCTGAGTACCGCTGAGGCGGGGCTACGACTTCGT 1284
Xe81-23     GATCCATTGCGCGGGATAGGTTCGGGTCTGAGTGCCGCTGAGGCGGGGCTACGACTTCGC 1017
            ******************************** ************************ 
XccA44      TATGAGTTCACCCGAAAGTTCGCTCCCTACATCGGCGTGGTGTACGAGCGCGCGTTTGGC 1188
Xsp1219     TATGAGTTCACCCGAAAGTTCGCTCCCTACATCGGCGTGGTGTACGAGCGCGCGTTTGGC 1344
Xe81-23     TATGAGTTCACCCGAAAGTTCGCTCCCTACATCGGCGTGGTGTACGAGCGCGCGTTTGGC 1077
            ************************************************************ 
XccA44      AATACCGCAGACATGCGACGCGAGCATGGCGAGTCCTTTGAAGACACGCGCTTGGTCATC 1248
Xsp1219     AATACCGCAGACATGCGACGCGAGCATGGCGAGTCCTTTGAAGACACGCGCTTGGTCATC 1404
Xe81-23     AATACCGCAGACATGCGACGCGAGCATGGCGAGTCCTTTGAAGACACGCGCTTGGTCATC 1137
            ************************************************************ 
XccA44      GGCCTTCGTACCTGGTTCTAA 1269
Xsp1219     GGCCTTCGTACCTGGTTCTAA 1425
Xe81-23     GGCCTTCGTACCTGGTTCTAA 1158
            ********************
```

Figure 4-9. Continued

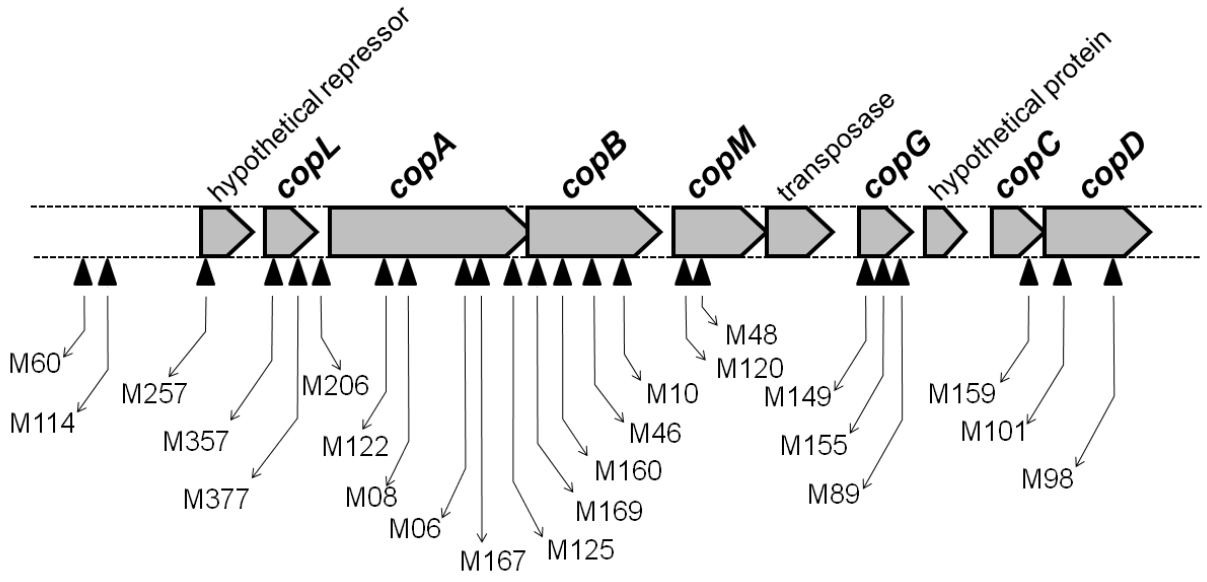

Figure 4-10. Transposon insertion sites within the copper resistance determinants of pXccCu2 from *Xanthomonas citri* subsp. *citri* strain A44. Black triangles indicate site of transposon insertion.

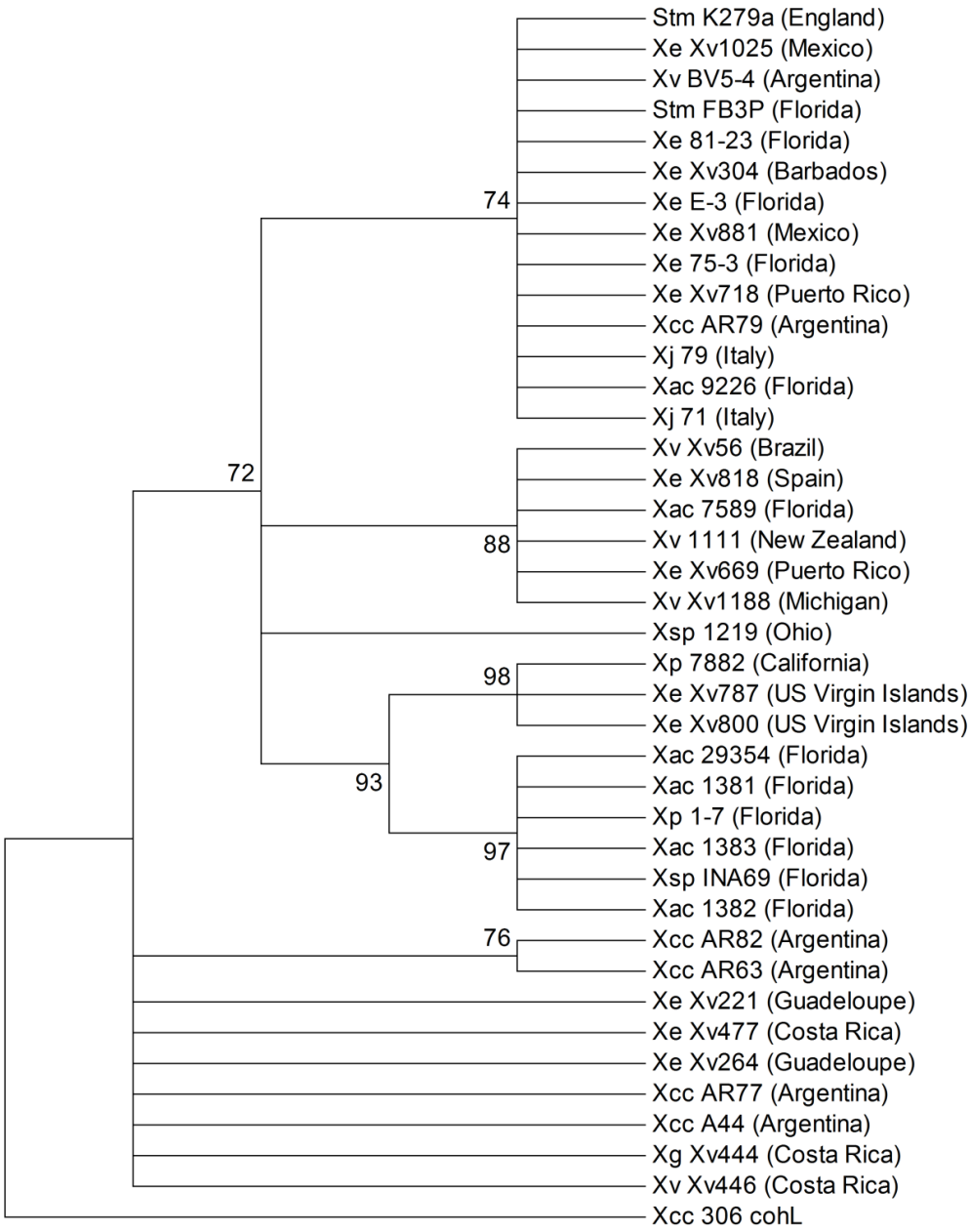

Figure 4-11. Phylogenetic tree constructed from alignments of partial nucleotide sequences of copper resistance gene *copL*, using the method of maximum parsimony. Bootstrap values, as percentage out of 1000 replicates, are shown at each node. Taxon information indicates organism, strain and geographical origin, respectively. Xcc, *Xanthomonas citri* subsp. *citri*; Xac, *X. alfalfae* subsp. *citrumelonis*; Xv, *X. vesicatoria*; Xe, *X. euvesicatoria*; Xp, *X. perforans*; Xg, *X. gardneri*; Xaj, *X. arboricola* pv. *juglandis*; Xsp, *Xanthomonas* sp.(1219, pathogenic; INA69, non-pathogenic); Stm, *Stenotrophomonas maltophilia.* Xcc 306 *cohL*, outgroup *copL* homolog gene from Xcc 306 present on the chromosome of copper resistant and sensitive *Xanthomonas* strains.

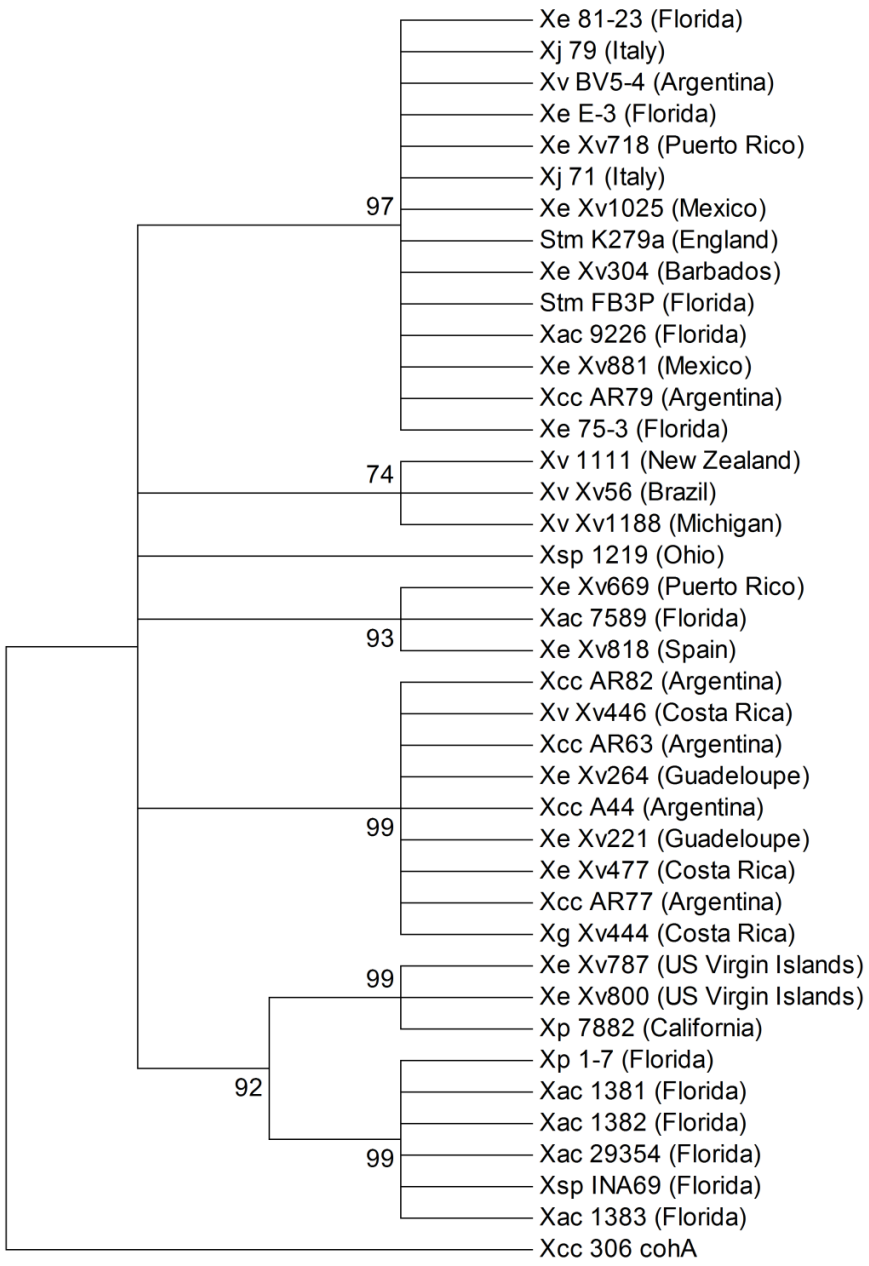

Figure 4-12. Phylogenetic tree constructed from alignments of partial nucleotide sequences of copper resistance gene *copA*, using the method of maximum parsimony. Bootstrap values, as percentage out of 1000 replicates, are shown at each node. Taxon information indicates organism, strain and geographical origin, respectively. Xcc, *Xanthomonas citri* subsp. *citri*; Xac, *X. alfalfae* subsp. *citrumelonis*; Xv, *X. vesicatoria*; Xe, *X. euvesicatoria*; Xp, *X. perforans*; Xg, *X. gardneri*; Xaj, *X. arboricola* pv. *juglandis*; Xsp, *Xanthomonas* sp.(1219, pathogenic; INA69, non-pathogenic); Stm, *Stenotrophomonas maltophilia.* Xcc 306 *cohA*, outgroup *copA* homolog gene from Xcc 306 present on the chromosome of copper resistant and sensitive *Xanthomonas* strains.

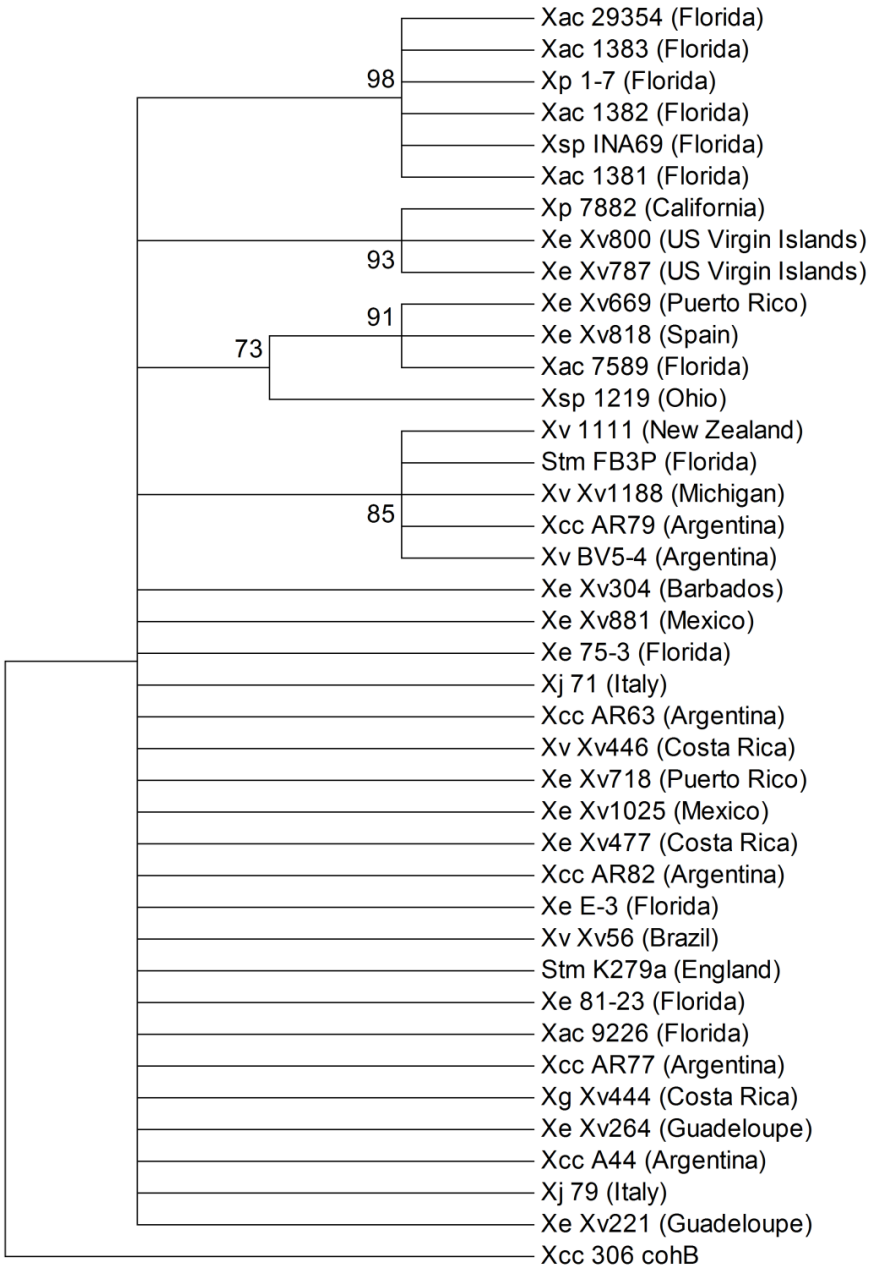

Figure 4-13. Phylogenetic tree constructed from alignments of partial nucleotide sequences of copper resistance gene *copB*, using the method of maximum parsimony. Bootstrap values, as percentage out of 1000 replicates, are shown at each node. Taxon information indicates organism, strain and geographical origin, respectively. Xcc, *Xanthomonas citri* subsp. *citri*; Xac, *X. alfalfae* subsp. *citrumelonis*; Xv, *X. vesicatoria*; Xe, *X. euvesicatoria*; Xp, *X. perforans*; Xg, *X. gardneri*; Xaj, *X. arboricola* pv. *juglandis*; Xsp, *Xanthomonas* sp.(1219, pathogenic; INA69, non-pathogenic); Stm, *Stenotrophomonas maltophilia.* Xcc 306 *cohA*, outgroup *copB* homolog gene from Xcc 306 present on the chromosome of copper resistant and sensitive *Xanthomonas* strains.

CHAPTER 5
SUMMARY AND DISCUSSION

After eradication efforts were suspended in Florida, attention has focused on alternative strategies to control citrus canker, including use of bactericides such as copper and streptomycin. One of the greatest concerns surrounding the use of these bactericides for control of citrus canker is that numerous sprays per season are usually necessary for efficacious disease control and frequent use may lead to development of resistant strains of the pathogen. Copper resistant (Cu^R) strains of *Xanthomonas citri* subsp. *citri* (Xcc) (syn. *Xanthomonas axonopodis* pv. *citri*), the causal agent of citrus canker, have been reported only in Argentina (Canteros, 1996). Streptomycin has not been used in commercial groves for control of citrus canker. Hence, the development of resistance to streptomycin in Xcc populations affecting citrus has not been reported yet. This antibiotic has been tested as a complementary measure to copper sprays (Graham et al., 2008). The purpose is to reduce the load of copper seasonally applied in citrus groves by replacing some copper applications by streptomycin or combining the two bactericides for higher effectiveness of control.

The major objectives of this dissertation were to assess the risk for the development of copper resistant (Cu^R) and streptomycin resistant (Sm^R) Xcc and to characterize and compare the genetics of copper resistance in Xcc with other bacteria.

None of the screened strains of Xcc from Florida and Brazil were identified as Cu^R. The strains from Brazil were isolated in 1996-1997, just a few years after the eradication program was replaced by an integrated management approach for citrus canker that includes, among other measures, the use of copper sprays to protect the foliage and fruit from damage (Leite and Mohan, 1990). Likewise, Xcc populations in Florida have

not been exposed to copper for a prolonged period. Moreover, samples of leaves with citrus canker collected in 2009 and 2010 from groves in Paraná did not reveal the presence of Cu^R strains in that area. Although this indicates that copper resistance in Xcc has either not yet developed or has not spread in the citrus growing areas of Parana or Florida, constant surveillance is advisable to assess the risk of copper resistance as long as copper sprays are repeatedly used in citrus groves with endemic canker.

Conversely, the majority of the *Xanthomonas alfafae* subsp. *citrumelonis* (Xac) strains screened in this study were identified as Cu^R. This is the first time copper resistance has been reported for Xac. Most likely, copper resistance has developed in Xac because citrus nurseries have been frequently sprayed with copper bactericides for control of citrus bacterial spot (CBS) from the time of eradication program in 1984 (Graham and Gottwald, 1991). Since Xac and Xcc share the same host and thrive under similar environmental conditions, the concern is that the interaction between these two bacteria in the mesophyll of leaves on newly planted nursery trees coinfected with CBS and citrus canker in citrus groves could result in horizontal transfer of Cu^R from Xac to Xcc.

In the present study, no Cu^R strain of Xcc was isolated from citrus trees sprayed with a copper bactericide every 21 days for 3 consecutive seasons. Due to the nature of the genetics of copper resistance in bacteria, which is conferred by several genes normally organized in operons (Cooksey, 1990; Mellano and Cooksey, 1988a; Voloudakis et al., 2005), a natural spontaneous mutation conferring copper resistance is unlikely to occur within bacterial populations. Conjugation of plasmid or transposable

elements carrying such resistance genes is likely to be the main means for enabling the development of copper resistance in bacterial populations (Bender and Cooksey, 1986; Bender et al., 1990; Stall et al., 1986). The relatively short period that Xcc population was exposed to copper during this study (3 seasons) and before that, due to the recent adoption of copper sprays for control of citrus canker in Florida after the eradication program was halted in 2006, may have accounted for the absence of Cu^R strains of Xcc in symptomatic trees repeatedly treated with copper in this study.

As observed for copper, no Sm^R strains were isolated after citrus trees had undergone 3 seasons of 21-day-interval sprays of streptomycin in the present study. Resistance to streptomycin develops either by horizontal transfer of resistance genes or by mutation (Gale et al., 1981; Springer et al., 2001). The latter is the more common mechanism of streptomycin resistance acquisition and occurs through a single base-pair mutation of the streptomycin binding site (Springer et al., 2001). Although Sm^R strains of Xcc were not found in the present study after 3 seasons of sprays, previous studies indicate that development of resistance in the Xcc population could occur any time. With continued use of streptomycin, resistance development is inevitable due to incessant mutation and selection in bacterial populations (Moller et al. 1981). What remains to be addressed is how likely Sm^R strains will develop in Xcc populations if only a few streptomycin sprays are intercalated or mixed with copper applications for control of citrus canker.

Although no Cu^R or Sm^R strains of Xcc were found, the frequent sprays of copper and streptomycin increased the population of epiphytic bacteria residing in the citrus phyllosphere resistant to these chemicals. The increased frequency is likely to reflect

changes in community structure, adaptation of the initial community as well as selection of resistant populations initially present. In the present study, total bacterial population in the phyllosphere did not differ between copper or streptomycin treated trees and untreated control. Therefore Cu^R and Sm^R bacterial communities may have taken over the sensitive ones, which were suppressed by the frequent bactericide sprays. Considering that cell density plays an important role in conjugation frequency (Levin et al., 1979; Normander et al., 1998), the concern for build-up Cu^R and Sm^R bacterial communities in the phyllosphere is that it increases the likelihood for exchange of resistance genes. Consequently, there is greater risk for the development of resistant strains of Xcc to these chemicals.

We showed that Cu^R genes can be transferred between different plant pathogenic species of *Xanthomonas* and that homologous of these resistance genes present in epiphytic bacteria residing on the citrus phyllosphere can confer copper resistance to sensitive strains of *Xanthomonas*. Despite that the movement of copper or streptomycin resistance genes from epiphytic strains to *Xanthomonas* could not be demonstrated in the present study, it is possible that in nature phyllosphere microorganisms represent a risk for the development of resistance in the Xcc population. In *Erwinia amylovora* mobilizable streptomycin resistance genes have been previously identified in common epiphytic bacteria found in orchards (Beining et al., 1996; Burr et al., 1988; Huang and Burr, 1999; Norelli et al., 1991; Sobiczewski et al., 1991). According to Sundin (2002), strA-strB genes can be carried within an integron, a transposon, or on broad-host-range plasmids. This genetic exchange has facilitated the world-wide dissemination of this determinant for streptomycin resistance among different bacterial genera (Sundin,

2002). Cooksey et al. (1990) reported the presence of Cu^R saprophytic *Pseudomonas putida* strains that harbor plasmid borne resistance genes homologous to those in *P. syringae* pv. *tomato* from a commercial tomato seed lot. The results reported here illustrate that copper resistance genes can potentially be shared between pathogenic *Xanthomonas* sp. and non-pathogenic epiphytic bacteria in the citrus phyllosphere.

This is the first time copper resistance has been characterized in Xcc and Xac strains. *copL*, *copA*, *copB*, *copM*, *copG*, *copC*, *copD*, and *copF* genes were identified in Xcc A44. The same *cop* genes except *copC* and *copD* occurred in Xac 1381. Comparison of copper resistance determinants in Xcc A44 and Xac 1381 to previously sequenced copper resistance determinats, such as Stm K279a (Crossman et al., 2008) and Xp 7882 (Voloudakis et al., 2005) revealed that high homology (≥92%) of nucleotide sequences is maintained among these strains only for *copLAB*, the N-terminal of *copM*, which is positioned immediately after *copB*, and *copF*, which is located at the end of the gene cluster in all strains. Although we could not determine the importance of *copF* for copper resistance by insertional mutation because this gene is absent in pXccCu2, we were able to demonstrate that the conserved region *copLAB* and part of *copM* has direct involvement in copper resistance. *copLAB* is essential for copper resistance and the N-terminal of *copM* is necessary for full resistance.

Homologues of the copper resistance genes *copLAB* cloned from Xcc A44 and Xac 1381 are present on the chromosome of Cu^R strains, such as Xv 1111 (data not published), and strains that have been tested to be Cu^S, such as Xcc 306 (da Silva et al., 2002) and Xv 85-10 (Thieme et al., 2005). Homologues of these genes are also present in many other *Xanthomonas* strains whose resistance or sensitivity to copper is

unconfirmed. The presence of homologues of copper resistance genes on the chromosome has been previously reported for other bacteria (Cooksey et al., 1990; Lim and Cooksey, 1993) and differently from what has been annotated, chromosomal *copLAB is* not responsible for copper resistance, but likely necessary for homeostasis and/or tolerance. While strains harboring the copper resistance genes *copLAB* highly similar (≥90%) to the ones cloned in this study can grow on MGY agar amended up to 400 mg L^{-1} of Cu, strains that have only the chromosomal *copLAB* genes, such as Xcc 306, grow up to 75 mg L^{-1} of Cu, hence, are Cu^S. Thus, to avoid further confusion or misinterpretation we suggest that the nomenclature of chromosomal homologues of *copL*, *copA* and *copB* in xanthomonads, which are probably copper homeostasis genes, should be changed to *cohL*, *cohA* and *cohB*, respectively.

Primers designed based on the A44 clone were used to PCR amplify *copL*, *copA* and *copB* from other copper resistant xanthomonads strains. All copper resistant and copper sensitive strains tested positive and negative with the three primer sets, respectively. Sequence alignments of *copLAB* genes from different strains indicated that the resistance genes are conserved among the Cu^R strains with identity of nucleotide sequences higher than 90%. Phylogenetic analysis revealed that the minor differences which exist in the nucleotide sequences of these strains are not related to the species or geographical origin. Xcc strains from Argentina were clustered into two different groups. Four strains were more closely related to strains of Xe, Xg and Xv from Costa Rica and Guadeloupe, and one Xcc strain was associated with an Xv strain also isolated from Argentina. This indicates that the copper resistance in xanthomonads may have a

common origin and that the Cu^R genes have been independently exchanged among different species of xanthomonads, possibly by horizontal transfer.

The presence of copper resistance genes from plant pathogenic xanthomonads in epiphytic bacteria such as *Xanthomonas* and *Stenotrophomonas,* as demonstrated in this study and the incessant movement of plant material, especially seeds, among countries may account for such wide dissemination of these genes into different *Xanthomonas* populations in different parts of the world, indicating a relatively high risk for copper resistance development in *Xanthomonas* pathogens under constant exposure to copper.

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BIOGRAPHICAL SKETCH

Franklin Behlau was born in Assis, São Paulo State, Brazil, in 1980. From 1999 to 2003 he attended the State University of Londrina, Londrina, Paraná State, Brazil, where he obtained the title of Agronomic Engineer. During this time, he got involved in research activities under supervision of Dr. Rui Pereira Leite at the Agronomic Institute of Parana (IAPAR), where he first started working with plant pathology. In 2004, he was admitted for the Master of Science program in plant pathology at Escola Superior de Agricultura Luiz de Queiroz – University of São Paulo (ESALQ/USP), Piracicaba, São Paulo State, Brazil, under the supervision of Dr. Armando Bergamin Filho. His master's research was focused on epidemiological studies of citrus canker on sweet orange trees under copper and windbreak protection. In 2006, he received an assistantship from the Citrus Research and Education Center (CREC), Lake Alfred, FL, to pursue a PhD degree in plant pathology at University of Florida, Gainesville, FL, where he conducted research on risk assessment of copper and streptomycin resistance development in *Xanthomonas citri* subsp. *citri* under supervision of Drs. James H. Graham and Jeffrey B. Jones.