Exploring the drivers of *Xanthomonas* population dynamics on tomato and pepper

By

Rishi Ram Bhandari

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Approved by

Neha Potnis, Assistant Professor of Plant Pathology
Leonardo De La Fuente, Professor of Plant Pathology
Mark R. Liles, Professor of Biology

Abstract

Bacterial leaf spot (BLS), caused by four species of *Xanthomonas*; *X. euvesicatoria*, *X. vesicatoria, X. perforans*, and *X. gardneri*, is an endemic disease of tomato and pepper in Southeastern United States and can result in total crop loss and there are no clear management strategies for this disease.

Host resistance is considered as most important and effective method of control of BLS disease. There are some resistant cultivars of peppers against BLS which but the occurrence of the pathogenic races overcoming the resistance genes in tomato resulted in no such resistant cultivars in tomato. Although chemical control based on copper bactericides has been used extensively since decades, occurrence of copper-resistant bacterial pathogens are reducing the efficacy of copper-based bactericides. As majority of antibiotics and heavy metal resistance genes are encoded in plasmids, frequent plasmid transfer among the strains plays an important role in spreading the resistance in field. Recently we found two copper resistant strains of *X. perforans* without these copper resistance plasmids and integration of copper resistance genes in the chromosome. The goal of this project was to identify the pathogen population diversity of BLS *Xanthomonas* in Alabama using culture dependent and culture independent (shotgun metagenomics) techniques. Moreover, we also studied the chromosomal copper resistance dynamics in *X. perforans* population and their transfer potential both *in planta* and *in-vitro* studies.

Sequencing and phylogenetic classification of 8 representative strains from the collection of 150 strains of BLS *Xanthomonas* from different places of Alabama suggest the presence of two novel sequence clusters within *X. perforans*. Culture-independent study of

diversity using shotgun metagenomics from tomato, pepper and weed samples showed *X. perforans* and *X. euvesicatoria* as a dominant pathogen in tomato and pepper respectively. Shotgun metagenomics also showed the co-infection by multiple species/genera in the field at a given time and provided strain-level resolution showing the presence of two or more lineages of *X. perforans* in tomato samples. The result further suggest that shotgun metagenomics can be used for higher resolution of pathogen population structure in diversity studies.

We also found copper tolerant *X. perforans* strains *Xp* 2010 and GEV 904 contained chromosomal encoded copper resistance genes in the genomic island. We further studied the structure of this island and investigate its transfer potential under *in-planta* and *in-vitro* conditions. Failure to obtain transconjugants/transductants under both conditions indicates that either the conditions we used for the transfer were not conducive or the transfer frequency is very low.

Overall, these study of the pathogen population dynamics helps in providing the insight of bacterial population situation in Alabama. Thus, protective measures can be taken before the outbreak of pathogen in a large scale. The long-term goal of this research is to improve our understanding about pathogen population in Alabama and pathogen dynamics in both host and non-host. This study will help the farmers and large-scale growers to select more resistant/tolerant cultivars and look forward for resistant breeding program based on pathogen population information.

Keywords: Xanthomonas, pathogen diversity, copper resistance, metagenomics, phage, integrative and conjugative elements

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1. CHAPTER ONE

Introduction and Literature Review

Introduction

Knowledge on pathogen population structure along with their genetic structure and dynamics is an essential factor for a successful management of plant pathogens (Burdon and Thrall 2008; Milgroom 2015). Agroecosystem provides a good environment for the pathogen to evolve rapidly because of interdependence of the pathogen and plants and selection pressure that has variety of implications in host pathogen co-evolution, shaping the pathogen population structure and speciation (Zhan et al. 2002). In agroecosystems, antibiotics, chemicals, host resistance and various cultural practices could be considered among the several factors that act as selective forces and contribute to pathogen evolution. The ability of the pathogen to overcome all these stresses and become successful in the plant environment depends upon its adaptive traits, virulence factors and evolutionary potential (McDonald and Linde 2002). Increased global movement of plant material, including seeds, transplants, nursery plants, can introduce exotic strains/species of the pathogen. Such invasion of novel variant/species into regions with endemic pathogens, could introduce diversity in the pathogen population (Brasier 2001).

Bacteria cause many economically important plant diseases, ranging from leaf spots, blights, wilt (Vidhyasekaran 2002; Mansfield et al. 2012). In the southeastern United States, there are multiple bacterial diseases, such as bacterial leaf spot on vegetables, ornamentals, canker on citrus, bacterial blight on cotton, prevalent on a variety of crops and vegetables. The pathogens responsible for these diseases have been persisting in this region over several

years due to lack of durable management strategies. The diversity arising in these endemic pathogens has been appreciated in the recent years, thanks to the next-generation sequencing technologies. Although majority of these endemic pathogenic bacteria have largely genetically monomorphic population structure based on multi-locus sequencing analyses (Timilsina et al. 2015; Parkinson et al. 2009; Wichmann et al. 2005; Ferreira et al. 2019), the variation arising in these pathogens has been majorly due to genetic processes such as horizontal gene transfer (via plasmids, phages, transposons, integrons, genomic islands) or homologous recombination (Achtman 2012; Linz et al. 2007; Maiden 2006; Ochman et al. 2000). These genetic variations can be hypothesized to be responsible for changes in the phenotypes, such as increased aggressiveness of pathogens, overcoming chemical/antibiotic resistance or host resistance, host jumps, etc. (Timilsina et al. 2019; Jibrin et al. 2018; Wiedenbeck and Cohan 2011; Dimitriu et al. 2015; Pál et al. 2005; Shapiro 2016; Chen et al. 2018).

There are several factors contributing to create diversity in pathogen population through these various events which mostly includes host selection pressure (Bartoli et al. 2016; Anderson and May 1982; Karasov et al. 2014; Wichmann et al. 2005), failure to follow recommended management practices such as overuse of pesticides, poor weed control, etc. (Sundin and Wang 2018; Stukenbrock and Bataillon 2012; McDonald and Stukenbrock 2016; He et al. 2016). In case of seed borne pathogens, multiple source of seeds or transplants along with the seeds from the previous season (Elmer 2001) also contribute towards introducing novel variants into the field. Spontaneous mutations and those in response to the environmental stress have also been reported to contribute to the diversity in plant pathogenic bacteria (Velásquez et al. 2018; Midha et al. 2017; Morris et al. 2009).

So, the central focus of this study is to identify drivers of diversity arising in the endemic bacterial plant pathogen. We have chosen bacterial leaf spot (BLS) *Xanthomonas* as

a model organism for the study of extent of variation arising in the endemic bacterial pathogen for many reasons that have been reviewed below.

Bacterial leaf spot of tomato and pepper: an endemic plant pathogen of Southeast United States

The genus *Xanthomonas* constitutes straight, gram-negative, yellow-pigmented, rod shaped bacterium with a polar flagellum. These bacteria are strictly aerobic chemoorganotrophs, phytopathogenic and fall under the division Gammaproteobacteria. They affect wide variety of crops, including solanaceous crops, *Citrus* spp., *Oryza* spp., crucifers, etc. where each individual species shows a high degree of host specificity (Hayward 1993; Chan and Goodwin 1999). As different genera within *Xanthomonas* display a narrow host range and are very homogeneous, the diversity within this genus is mostly governed by host range and pathogenicity (Dye et al. 1980).

Bacterial leaf spot pathogen (BLS) has been identified as a destructive disease of tomato and pepper throughout the world (Hamza et al. 2010; Potnis et al. 2015). It was first identified as *Bacterium vesicatoria* in South Africa in 1914 (Doidge 1921). Later, these bacteria were classified as *Xanthomonas vesicatoria* and then as *X. campestris* pv. *vesicatoria* based on their host specificity (Young et al. 1978). During 1990s, (Vauterin et al. 1995) and (Stall,' et al. 1994) identified that two genetically and phenotypically distinct group (group A and B) exist within *X. campestris* pv. *vesicatoria* which was reclassified into two groups, *X. axonopodis pv. vesicatoria* (group A) and *X. vesicatoria* (group B). In 1957, (Sutic 1957) identified a bacterium in tomato in the former Yugoslavia and named it *Pseudomonas gardneri*. However, based on comparison with several other *Xanthomonas,* (Dye 1966) found this strain similar to *X. vesicatoria*. Later (Jones 1995) characterized this strain and another strain from Florida as additional C and D groups. Based on DNA-DNA hybridization, the Group C strains from Florida was found

closely related to A and placed under *X. axonopodis* pv. *vesicatoria,* whereas the strains originally identified by (Sutic 1957) (group D) were genetically distinct and was later reclassified as *X. gardneri* (Dye 1966). Later based on the result of DNA-DNA hybridization, (Jones et al. 2004) showed that group C and D are distinct species and the disease is caused by four different species; *X. euvesicatoria* (A), *X. vesicatoria* (B), *X. perforans* (C), and *X. gardneri* (D). In the United States, *X. perforans* and *X. euvesicatoria* are dominant species pathogenic on tomato and pepper (Schwartz et al. 2015). *X. gardneri* is reported in Michigan, Indiana and Pennsylvania (Ma et al. 2011).

Identification of different *Xanthomonas* species and lineages within a single species can be done by multi locus sequence analysis (MLSA) based on six housekeeping genes (*fusA, gapA, gyrB, lacF, and lepA*) (Almeida et al. 2010). MLSA studies of different *Xanthomonas* species has shown several recombination events between distinct plant pathogenic lineage that leads to the generation of novel variants with different pathogenicity and virulence factors (Jibrin et al. 2018; Timilsina et al. 2015). Different secretion system (type II, III, IV, V, VI) present in BLS *Xanthomonas* secrete different effectors, cell wall degrading enzymes, virulence proteins and various mechanism of host pathogen interaction that are responsible for pathogenicity and host specificity in BLS pathogen (Potnis et al. 2011; Thieme et al. 2005; Grant et al. 2006; Souza et al. 2011; Singer et al. 2013; Kim et al. 2013).

Pathogen population diversity of BLS Xanthomonas in Florida

BLS pathogen has shown a great diversity in its population structure in Florida where this pathogen has been studied extensively. Prior to 1991, only *X. euvesicatoria* was a dominant pathogen of tomato in Florida. During 1991, *Xanthomonas perforans* tomato race 3 (T3) strain was first identified which then took over *X. euvesicatoria*. By 1998, there was a race shift where *Xanthomonas perforans* race 4 was first identified without any host selection

pressure, i.e.; even in the absence of any resistance genotypes of tomato against BLS. In 2006, there was presence of two genomogroups within *X. perforans*. Among the two genomogroups, one of the genomogroups showed the signature of recombination events originating in *X. euvesicatoria*. Later by 2012, the population of *X. perforans* changed from T3 to T4. Three different mutations were observed in the *avrXv3* gene of T4 strains. The pathogen population acquired *avrBsT*, which was first observed in 1998, in most strains in 2006, and in all strains examined from 2012. Although *Xanthomonas euvesicatoria* was the dominant pathogen of pepper, in 2010, *X. perforans* was isolated from infected pepper samples, suggesting possible host range expansion (Timilsina et al. 2015; Schwartz et al. 2015). Outbreaks of *X. vesicatoria* or *X. gardneri* have not been reported in the United States (Timilsina et al. 2015).

BLS disease symptoms, epidemiology and disease cycle

Disease symptoms of BLS can be found on leaves, fruit, flowers and stalk of tomato and pepper plants. Initially, small dark brown water-soaked spots appear as light to dark green areas on the young infected leaves (Figure: 1-1). The individual lesions develop rapidly in size and appear black and greasy in tomato and tan and brownish red in pepper (Sun et al. 2002). Lesion are irregular and generally with or without yellow halo. Later, the lesions coalesce to form large, blighted areas on leaves. The blighted leaf decrease the net photosynthetic area and causes premature leaf fall (Vallad et al. 2016; Sun et al. 2002). Lesions are generally more numerous on young tissue and defoliation is most common in pepper leaf whereas the tomato leaf remains scorched in the plant. In the nursery and transplant house, affected seedlings become more spotted with leaf yellowing and in severe stages with defoliation (Cerkauskas 2005). The lesions on infected stems and petioles are similar and elliptical in shape. Fruit spots first appear as small pale green raised areas surrounded by water-soaked borders, which eventually become brownish, raised and scabby.

Although being small and not too deep, large numbers of these spots will lower the quality of the fruit. Similarly, the collapsed lesions may harbor large number of fungus and other bacterial that might cause several diseases.(Sun et al. 2002).

Disease development is favored by temperatures of 75 to 86˚F depending on the particular bacterial species, relative humidity above 85%, and extended periods of leaf wetness (Araújo et al. 2011). Relative humidity below 40% for short period will reduce disease severity and development. Night temperature below 61˚F suppress the disease development (Boucher 2012). Bacteria can move within fields by wind-driven rain, irrigation droplets, aerosols, and the handling of wet plants.

The disease is seed borne and originates from infested seeds. Once the bacteria reaches the leaf surface, it colonizes the phyllosphere (Potnis et al. 2014). The pathogen survives epiphytically on the transplants and often passes asymptomatically through the transplant houses. Upon the favorable environment of high humidity, temperature and host, bacteria enter plant apoplast through natural openings and wounds. High pressure mist and blowers also increase the number of entry sites for infection (Boucher 2012). Once inside the plant cell, bacteria secretes different effectors to acquire nutrients (Zhang et al. 2009; Potnis et al. 2015). When the epidermal cells ruptures, these bacteria are released again which serve as a secondary inoculum and is then carried by various sources to other healthy tissues (Boch and Bonas 2010). In the field, pathogenic *Xanthomonas* can overwinter in host debris and phyllosphere of its hosts and non-hosts which can also acts as a source of inoculum. (Schaad and White 1974; Leben 1981). A brief disease cycle of bacterial leaf spot pathogen of tomato and pepper is given in figure 1-2.

From the disease cycle we could see that, there are several points where the pathogen can get introduced in the field. For e.g., the grower can have infected seed, they could have

the transplants from different sources where they could get different pathogens, they could have the pathogen from the previous season overwintering in the infected debris or surviving in the alternate host or it could be rain or wind driven from neighboring field. So, within a single field we could have a multiple source of inoculum therefore there could be multiple genotypes co-existing within a single field.

Control strategies for BLS Xanthomonas

Higher multiplication rate of pathogen, mutation and acquisition of gene through gene transfer, and lack of effective management strategies are the major challenges to control BLS for many growers all over the world (Obradovic et al. 2008). Although there is no viable option for the management of this disease, integrated disease management strategies helps in the reduction of disease incidence significantly in the field.

Host resistance is considered as most important and effective method of control of BLS disease. Six hypersensitive resistance genes, namely *Bs1, Bs2, Bs3, Bs4, Bs7, BsT* and two recessive genes governing non-hypersensitive resistance, *bs5* and *bs6* has been reported in pepper and most of the commercial cultivars tries to incorporate these genes for BLS resistant pepper (Kim and Hartmann 1984; Cook and Guevara 1984; Potnis et al. 2012; Jones et al. 2002; Vallejos et al. 2010; Minsavage 1990a). The hypersensitive reaction in pepper plants is induced by the interaction of these *Bs* genes with their corresponding avirulence (*avrBs*) genes. Occurrence of the pathogenic races overcoming the HR genes has resulted in the inability to control the disease (Potnis et al. 2012; Minsavage 1990a). In tomato, four sources of resistance have been identified; *Rx1, Rx2, Rx3* (Yang et al. 2005), *Bs4* (Bonas et al. 1993), *Xv3* (Astua-Monge et al. 2000a) and *RXopJ4* (Sharlach et al. 2013). Continuous change in pathogen population structure of BLS *Xanthomonas* over time in Florida has resulted in overcoming the resistance against race T1 and T3 (Minsavage et al. 2003; Astua-Monge et al. 2000b; Pei et al. 2012; Scott et al. 2001). Later race T4 strains with moderate to

high level of conserved *XopJ4* effector was seen in Florida (Sharlach et al. 2013). Although *RXopJ4* introgressed tomato lines are found to be effective against BLS (Bhattarai et al. 2017), these plants showed low fruit yield, small fruit size and autogenous leaf necrosis (Sharlach et al. 2013). Developing BLS resistant cultivars of tomato based on targeted genome editing can be a helpful resource to fight against BLS in the future (Sharma and Bhattarai 2019). Currently, there are no commercial tomato cultivars available with resistance to BLS.

Given the emergence of streptomycin resistant *Xanthomonas* strains, it is no longer used to control BLS pathogen (Stall and Thayer 1962). Copper based bactericides and antibiotics are being widely used as a chemical control strategy in BLS (Jones et al. 1991; Higgins 1922). Copper hydroxide and copper sulfate-based bactericides mixed with mancozeb helps in increasing the active copper ion concentration for better disease control (Marco and Stall 1983). However, extensive use of copper-based bactericides has also led to the existence of copper resistance strains. Copper resistance in BLS was first observed in 1968 (Marco and Stall 1983; Stall et al. 1986). In the recent days, more research has been done to understand the effect of nanomaterials to control pathogen (Servin et al. 2015). Although photocatalytic crystalline nano particles are found to have a greater ability to control disease as compared to copper bactericides, phytotoxicity was the major problem associated with their efficiency (Paret et al. 2013).

Similarly, alternative disease control strategies for bacterial spot of tomato include activation of natural plant defense mechanisms by systemic acquired resistance (SAR) inducers. Inducers such as Acibenzolar-S-Methyl (Romero et al. 2001), both harpin and acibenzolar-S-methyl (Obradovic et al. 2005; Louws et al. 2001) are found effective against this disease but negative impact on plant growth or yield have been reported (Csinos et al. 2001; Romero et al. 2001). Plant growth-promoting rhizobacteria (PGPR) induced systemic

resistance (ISR) (Kloepper et al. 1999; Zehnder et al. 2001) and direct mechanism by improved acquisition of nutrients and production of phytohormones (Podile and Kishore 2007; Sashidhar and Podile 2010) has been studied as an effective control agents against several plant pathogens. Application of antagonistic microbes in the phylloplane have shown varying efficacies for bacterial spot control (Roberts et al. 2008; Moss et al. 2007; El-Hendawy et al. 2005; Hert et al. 2009). Similarly, several saprobe fungi has been proved to be beneficial to control BLS in tomato in Brazil (Cristina Martins Barros et al. 2015).

Transgenic approaches transferring resistance gene *Bs2* from pepper and *EFR* genes from *Arabidopsis* in tomato controls bacterial spot and bacterial wilt in tomato to a significant level (Kunwar et al. 2018). Beside traditional breeding, various genome editing tools like TALENs (Transcription Activator Like Effector Nucleases) and ZFNs (Zinc Finger Nucleases) are being investigated to develop bacterial leaf spot cultivars (Joung and Sander 2013). CRISPR/Cas9 mediated mutagenesis in tomato helped in acquiring broad spectrum diseases resistance (Thomazella et al. 2016).

Bacteriophages has been extensively used for the control of plant disease (Obradovic and Jones 2004; Buttimer et al. 2017; Jones et al. 2007). Phages are viruses that specifically infect bacteria and have no direct negative effects on animals or plants. Several greenhouse and in field experiments with bacteriophages has shown a better disease control as compared to chemical controls (Balogh et al. 2003; Obradovic and Jones 2004; Flaherty et al. 2000). However the use of bacteriophage as an effective phage therapy strategy to control bacterial leaf spot faces significant challenges (Jones et al. 2012). The phyllosphere environmental factors such as rain, sunlight, temperatures, pH, etc. plays an important role for survival of phages (Civerolo and Keil 1969; Balogh 2002; Iriarte et al. 2007). Use of integrated disease management strategies with systemic acquired resistance inducers, biocontrol agents

integrated with conventional practices and chemical methods, represent new quality in plant protection and increased efficiency of the disease management (Obradovic et al. 2008).

Antibiotic and copper resistance in BLS Xanthomonas

During 1950s, streptomycin sprays was commonly used to control bacterial disease, including bacterial spot. Later the antibiotics was no longer used due to the development of streptomycin resistant strains of *Xanthomonas euvesicatoria* (Race T1), the dominant pathogen of BLS in Florida (Stall and Thayer 1962; Minsavage 1990b). In the 1960s, fixed copper compounds and copper-fungicide mixes became the primary means of bacterial spot control.

Later in 1960s, fixed copper compounds and fungicides became the major option for chemical control of bacterial and fungal plant pathogens. These compounds have enhanced effectiveness when used in combination with ethylene-bis-dithiocarbamate (EBDC) fungicides (e.g. maneb or mancozeb) based on the increased availability of free Cu^{2+} ions (Marco and Stall 1983). However widespread use of copper has created a selection pressure for the bacteria as a result of which copper-resistant bacterial pathogens have become prevalent and have reduced the efficacy of copper-based bactericides (Adaskaveg 1985; Bender and Cooksey 1986a, 1987; Cooksey et al. 1990; Lee et al. 1994; Marco and Stall 1983; Sundin et al. 1989). Most of the genes associated with copper resistance from plant-pathogenic bacteria are plasmid encoded (Bender and Cooksey 1986a; Bender,' et al. 1990; Cooksey 1987, 1990; Stall et al. 1986; Voloudakis et al. 2005). Genes namely *copL, copA, copB, copM, copG, copC, copD,* and *copF* present in copper resistant *Xanthomonas* are responsible for the resistance and copper homeostasis or tolerance (Crossman et al. 2008; Potnis et al. 2011). Copper-resistant strains of BLS pathogen exist worldwide in several geographical regions (Marco and Stall 1983; Jones et al. 1991; Ritchie and Dittapongpitch 1991). Even a partial population of bacterial strains showing copper resistance should be of

concern, as resistance can develop in a whole population by exchange of copper resistance genes between strains (Cooksey 1990). Copper resistance genes have been found in copper-resistant strains of *Xanthomonas* and other bacteria (Behlau et al. 2013). In most cases, these genes have been located on self-transmissible plasmids (Stall et al. 1986) but gene clusters associated with the chromosome in a single strain XVP26 have been found as well (Basim et al. 2005). Mobilization of plasmids between copper-resistant and copper-sensitive strains has been demonstrated under laboratory conditions, and this transfer of plasmids also occurs in nature (Canteros et al. 1995).

BLS pathogen dynamics has been studied extensively in Florida. Previous studies based on whole genome sequencing of randomly selected pathogen isolates from different fields in the Southeastern and Midwest United States have shown dominance of *X. perforans*, *X. euvesicatoria* and *X. gardneri* respectively, with *X. perforans* exhibiting extensive diversity. However, there often lies the bias of selecting random isolates in such studies that might underestimate the diversity existing in the fields. Although *X. gardneri* has not been detected in Southeastern United States, it is a major concern, and could, if introduced, undermine resistance genes currently deployed for the management of *X. euvesicatoria* in pepper. Although extensive studies on BLS pathogen population dynamics were done on Florida, we don't know the status of this pathogen population in its neighboring states. As there are several transplants houses where they share transplants between these states, we assume a pathogen population similar to that of Florida in other neighboring states.

The development of effective management strategies, primarily the selection of resistant plant material, relies upon the accurate identification of pathogens and an understanding of their diversity and pathogenicity. Though outbreaks of BLS occur in most commercial growing regions in Alabama, little has been done to investigate the genetic diversity and distribution of the causal *Xanthomonas* species. This study describes the

identification and diversity of pathogenic *Xanthomonas* sp. associated with BLS in both host and non-host in Alabama based on molecular analysis. So, the comprehensive research in the pathogen population dynamics of BLS in Alabama helps to understand the pathogen situation and possibility of significant outbreaks in the future. Thus, various protective measures and breeding program can be directed regarding existing pathogen.

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Figure 1-1: Bacterial leaf spot symptoms in tomato (left) and pepper (right) leaves showing characteristics necrotic lesions.

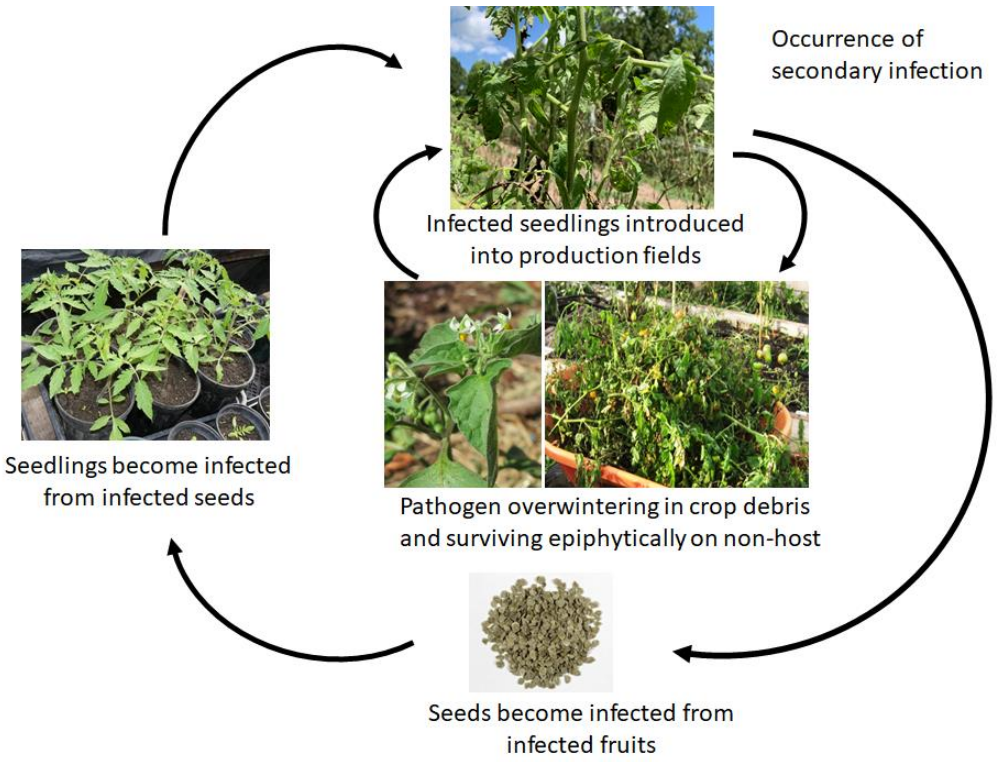

Figure 1-2: Disease cycle of BLS *Xanthomonas*

2. CHAPTER TWO
Population structure of BLS *Xanthomonas* in Alabama

Abstract

Bacterial leaf spot (BLS) of tomato and pepper is an endemic pathogen to the Southeast United States. The disease is caused by four distinct species of *Xanthomonas*; *X. perforans* (*Xp*), X. *euvesicatoria* (*Xeu*), *X. gardneri* (*Xg*) and *X. vesicatoria* (*Xv*). In the Southeast, *X. perforans* is a dominant pathogen of tomato and *X. euvesicatoria* is responsible for the disease in pepper. BLS pathogen has undergone a great shift in pathogen population structure in Florida suggesting the evolution potential of this pathogen. Lack of effective management strategies has been a continuing problem for the growers. A diverse pathogen population of *Xanthomonas* would respond differently to different control strategies. So, generalizing management strategies across southeastern United States might not be effective. This study involves culture dependent and culture independent (shotgun metagenomics) approach to study the pathogen population structure of BLS in Alabama. From 150 strains of BLS *Xanthomonas* collected in the year 2017 and 2018 from different places of Alabama, we sequenced 8 representative *Xanthomonas perforans* strains by illumina MiSeq Micro platform. Phylogenetic classification of these *perforans* strains along with other strains collected around the world suggest the presence of two novel sequence clusters within *X. perforans* population from Alabama. Presence of *X. perforans* in pepper samples from Alabama suggest host range expansion. Culture-independent study of diversity using shotgun metagenomics of 15 tomato, pepper and weed samples predict *Xanthomonas perforans* and *X.*

euvesicatoria as a dominant pathogen in tomato and pepper respectively. The metagenomics analysis also showed the presence of *X. perforans* in pepper sample suggest the host range expansion and *X. perforans* might be an emerging pathogen in pepper. Shotgun metagenomics provided strain-level resolution with presence of two or more lineages of *X. perforans* in tomato samples. We also observed presence of co-infection by multiple species/genera in tomato/pepper samples suggesting shotgun metagenomics can be used for higher resolution of pathogen population structure in diversity studies.

Introduction

With the advancement of new sequencing technologies, genomics techniques are being extensively used in taxonomy to differentiate different bacterial species and are highly correlated with standard DNA-DNA hybridization technique (Xu and Wang 2019). Whole genome sequencing based on rapid and inexpensive next generation DNA sequencing technology is being widely used for population genetics to explore the genetic similarity and difference within and between populations. This powerful tool can be used to reveal the pathogen adaptation, speciation, population structure, diversity and evolution (Vinatzer et al. 2014). Studying of the pathogen population structure based on the sequencing result of few selected representative strains cannot be the true prediction of diversity as there is always a bias in selecting these representative strain. Shotgun metagenomics approach is a culture-independent and untargeted sequencing of all microbial genomes present in a sample bypassing the selection of representative strains from the sample (Bragg and Tyson 2014). It allows the recovery of whole genome sequences and can be used to profile taxonomic composition and functional potential of microbial communities (Quince et al. 2017). Shotgun whole metagenome sequencing (WMS) allows the identification of microbial taxa comprising a community to a higher resolution than 16S rRNA-based sequencing. Results of

these studies have revealed significant diversity, and many novel species/phylotypes have been identified (Becker et al. 2002; Corby et al. 2005).

Bacterial leaf spot (BLS) of tomato and pepper is an economically important disease of both processing and fresh market fruits that can affect all aerial parts of the plant and occurs in the tropical, subtropical, and temperate regions of world (Jones et al. 2004; Hamza et al. 2010). In the United States, BLS of tomato and pepper is an endemic disease and has been problematic in the Southeast. The disease is caused by the genetically diverse group of *Xanthomonas*; *X. euvesicatoria, X. vesicatoria, X. perforans*, and *X. gardneri* (Jones et al. 2004). These 4 species were previously designated as genetic groups A, B, C, and D and they were differentiated based on their phenotypic and genotypic traits (Jones et al. 2004). All four species of bacterial spot pathogen affect all aerial parts of tomato and pepper by causing leaf spots, defoliation, and fruit lesions (Jones et al. 1991). These pathogens can survive as an epiphytes on tomato foliage, volunteer plants, weeds, and diseased plant debris (Jones et al. 1986; Cuppels et al. 2008). BLS is a seed borne pathogen and are spread through contaminated seeds and transplants as a primary source of inoculum (Dutta et al. 2014). Groups A and B overwhelmingly constitute the largest number of strains that infect tomato and pepper worldwide (Bouzar et al. 1994). However, widespread regional specialization for each group has also been reported (Bouzar et al. 1996, 1999; Sahin and Miller 1996).

Studies on BLS pathogen population structure from Florida has shown a great diversity in pathogen population where the pathogen has undergone species wipeout, race shift and host range expansion (Timilsina et al. 2016; Schwartz et al. 2015). Multilocus sequence analysis (MLSA) studies based on six housekeeping genes showed the presence of two phylogenetic groups within *X. perforans* strains collected in Florida. Among these two group, one of the group showed the signatures of recombination events originating in *X.*

euvesicatoria (Timilsina et al. 2015). Genomics study based on core genome MLSA suggested multiple recombination events responsible for the change in pathogen population in Florida (Timilsina et al. 2019). The study on pathogen population structure of *X. perforans* in Florida was based on whole genome sequencing of the isolates that was collected over time. Based on 34 isolates collected in the span of around 20 years, we have seen a great diversity in pathogen population.

Although extensive studies in the pathogen population is done in Florida, little has been known about the pathogen in other neighboring Southeastern states. As we know that there are multiple transplant houses in these states where they transfer the transplants between the state, management strategies for this pathogen in this region has been generalized and is followed based on the pathogen population structure of Florida. Though outbreaks of bacterial spot occur in most commercial growing regions in Alabama, little has been done to investigate the genetic diversity and distribution of the causal *Xanthomonas* species. The development of effective management strategies including resistance breeding, relies upon the accurate identification of pathogens and an understanding of their diversity and pathogenicity. This study describes the identification and diversity of pathogenic *Xanthomonas* sp. associated with BLS in both host and non-host plant in Alabama based on molecular analysis. Thus, various protective measures and breeding program can be directed regarding existing pathogen. Moreover, from the population diversity studies of *X. perforans* from Florida, we have noted recombination events in *Xp* lineages with origin in *Xeu*. So, there could be a possibility of *Xeu* existing together with *Xp* in low proportions that cannot be detected by culture-dependent methods. To answer this question we, decided to test the proof of concept that shotgun metagenomics can reveal presence of co-infecting lineages/species that could explain different recombination/horizontal gene transfer (HGT) patterns we have

been observing and that have been responsible for changes in the pathogen population structure.

Materials and methods

Bacterial strain collection, sequencing, and assembly for culture-based population diversity of Xanthomonas in Alabama

From 150 *Xanthomonas* strains collected from symptomatic tomato and pepper in the year 2017 and 2018 from different places of Alabama, eight bacterial strains (Table 2-1) were selected randomly for draft genome sequencing. Genomic DNA was extracted using CTAB-NaCl method as described by (Howland 1996) with some modifications. The degradation and contamination of the genomic DNA was monitored on 0.5% agarose gels and DNA concentration was measured using Qubit® DNA Assay Kit in Qubit® 2.0 Flurometer (Life Technologies, CA, USA) and submitted to the Georgia Genomics and Bioinformatics Core, University of Georgia for library preparation and sequencing. Paired-end reads were generated by multiplexing 12 libraries in a single lane on an Illumina MiSeq Micro (PE150) platform. Sequence adapter filtering, quality trimming and contaminant removal was performed using BBDuk (https://jgi.doe.gov/data-and-tools/bbtools/bb-tools-user-guide/bbduk-guide/). De novo assemblies were constructed with SPAdes version 3.5.0 (Bankevich et al. 2012) with kmers of 127, 117, 107, 97, 87, 77, 67 using the 'careful' flag. Genome statistics including GC content, contig number, N50 and genome length were calculated with QUAST web interface (Table 2-1) (Gurevich et al. 2013).

Reconstruction of the X. perforans core genome

The sequencing reads for 45 *X. perforans* strains downloaded from the NCBI (Table 2-2) along with quality-trimmed reads generated in this study and were individually aligned against the completed genome of *X. perforans* strain 91-118 using the Burrows-Wheeler

Alignment tool (v.0.7.17-r1188, 46). The reads with a mapping quality of 60 (i.e., uniquely 368 mapped reads) and Phred quality score of 20 (at least 99% consensus) were included in the analysis. Variants were called from the whole genome alignments using FreeBayes (v.1.2.0, 48) and single-nucleotide polymorphisms (SNPs) common to all genomes were extracted to generate a concatenated set of high-quality core-genome SNPs using Parsnp (Treangen et al. 2014). The phylogenetic tree was visualized and annotated using FigTree (v.1.4.2, http://tree.bio.ed.ac.uk/software/figtree/).

Sample collection and DNA extraction for culture independent method to understand the population diversity of Xanthomonas in Alabama

Samples were collected from symptomatic tomato, pepper plants and volunteer weeds, from different Alabama counties during the summer of 2017 and 2018 (Table 2-3). About 10 grams of the sample was taken in Ziploc bag (Ziploc®) with 50 ml 0.1% wash buffer (0.05M PBS, 8.5 g NaCl and 0.2ml tween 20 per liter of water). The sample were then sonicated for 15 minutes followed by transfer of the buffer to a 50 ml falcon tube with the help of sterile pipette. The tubes were then centrifuged at 4000 rpm, 4°C for 20 minutes (Eppendorf® 5418 R) to collect all the cells followed by removal of buffer without disturbing the pellet at the bottom. The cells were then washed twice with distilled water and extraction of total DNA were done using Wizard® Genomic DNA Purification Kit (Promega) as per the manufacturer's instruction with few modifications. The extracted DNA was quantified using nanodrop and kept at -20^{0}C until further processing. Sequencing was performed on Illumina HiSeq 4000 platform. The raw reads were processed and trimmed for adapter, quality and contaminant using BBDuk (https://jgi.doe.gov/data-and-tools/bbtools/bb-tools-user-guide/bbduk-guide/). Similarly the host reads were separated and removed from the metagenomic samples using KneadData tools

(https://bitbucket.org/biobakery/kneaddata/wiki/Home). Then the quality trimmed reads were processed using the MOCAT pipeline (Kultima et al. 2012) to determine bacterial species abundance under the metagenomic Operational Taxonomic Unit (mOTU) framework. Alignment of the metagenomic reads against the reference database of phylogenetic marker containing 2,494 metagenomic mOTUs (meta-mOTUs) and 5,232 reference mOTUs (ref-mOTUs) results in classification of prokaryotic organisms at the species level (Sunagawa et al. 2013).

Databases preparation and metagenome read alignment for strain level profiling of metagenomic samples

To investigate the intraspecific diversity and dynamics of *X. perforans* within the metagenomics samples, we used the newly developed StrainEst pipeline (Albanese and Donati 2017). StrainEst is a reference-based method that uses single-nucleotide variant (SNV) profiles of selected reference genomes to estimate the presence and relative abundance of strains within a sample. Core genome alignment of SNPs from *X. perforans, X. euvesicatoria* and its sister clade which includes *X. alfalfae*, *X. citrumelonis* and related strains was done using Parsnp (Treangen et al. 2014). Bayesian statistical clustering of core genome alignment was done using hierBAPS to show the distinct sequence clusters (SCs) with in the population (Cheng et al. 2013).

A representative strain from each SC was selected and aligned against the completed reference genome of *X. perforans* strain 91-118 using the MUMmer algorithm (Kurtz et al. 2004). This reduced genome alignment was used to construct a SNV matrix, where each row corresponded to a variable position in the reference genome and columns contained allelic variants present in the reference strains. This matrix was used as a reference in the modeling of strain level abundance as described below. For each metagenome, the quality trimmed reads were mapped to the representative *X. perforans* genomes using Bowtie 2 (Langmead

and Salzberg 2012) and the frequency of variant sites at each position in the SNV matrix were extracted. Positions with a depth of coverage lower than the 10th percentile and higher than the 90th percentile were filtered from the analysis and reference genomes were required to display greater >95% alignment coverage with >1% relative abundance to be considered present in a sample. Finally, a Lasso regression between the measured and predicted allelic frequencies at SNV positions was used to infer the relative abundance of *Xanthomonas* sp. in the metagenomic samples. Pearson's correlation coefficient was calculated to assess the quality of the model prediction.

Results

Diversity of BLS reveals presence of novel sequence clusters in *X. perforans* population in Alabama

Reconstruction of the *X. perforans* (*Xp*) core genome reveals the presence of two novel genetic clusters composed of *Xp* strains collected in Alabama. A maximum likelihood phylogeny constructed from a concatenated alignment core-genome single nucleotide polymorphisms (SNPs), coupled with a Bayesian analysis of population structure, revealed the presence of seven distinct SCs within *X. perforans* (Figure 2-1). SCs 1 through 4 corresponded to the previously described population structure of *X. perforans* strains collected in Florida, while SCs 5 and 6 composed Alabama strains sequenced in this study. The SC 7 is composed of the *X. perforans* strains collected from tomato in Australia (Roach et al. 2018). One Alabama strain (ALS7E) isolated from pepper clustered in SC 3 along with other strains from Florida, while the remaining pepper strains (ALS7B, AL65, and AL66) forms a novel SC 6 along with one tomato strain AL1. Similarly, one Alabama strain AL57 grouped with other *X. perforans* strains collected in Florida within SC4.

Microbial taxonomic abundance based on mOTU reveals mixed infection with different microbial genera

Operational and taxonomic unit (OTU) clustering from mOTU pipeline showed the presence of *Xanthomonas* species complex dominant in most of our metagenomics sample collected in both years except the pepper sample Pep E (18) (Figure 2-2). This sample had a characteristic water soaking phenotype during the time of sampling which we thought to be *Xanthomonas,* but the taxonomic classification does not show any *Xanthomonas* associated in the sample. The microbial heatmap shows the presence of several rot causing pathogen such as *Dickeya* sp, *Pantoea* sp. etc. which could be responsible for such phenotype. In some of our samples, we saw the presence of *Pseudomonas cichorii* and the abundance of this pathogen was higher than *Xanthomonas* complex in tomato sample Tom E (17). Most of our samples showed the abundance of nonpathogenic *X. arboricola* and *Pseudomonas* sp. From our single sample from weed (Weed C (18)), we also saw the presence of *Xanthomonas* species showing the survival of these pathogenic *Xanthomonas* in the weed.

Taxonomic clustering from mOTU showed the mixed infection and it cannot distinguish between the closely related species, *Xeu/Xp,* thus, unable to assess presence of multiple closely related species/lineages in the sample.

Strain level resolution of metagenomic samples reveals the presence of more than one SCs and co-infection with different species in the same field

Ninety-six draft genome sequences of *X. perforans, X. euvesicatoria* and its sister cluster downloaded from GenBank were used to construct the core genome alignment of SNPs using Parsnp. The resulting alignment subjected to Bayesian statistical clustering using hierBAPS showed the presence of four distinct sequence clusters within *Xanthomonas* species complex (Figure 2-3). The representative strains from each sequence cluster (Table 2-4) form hierBAPS result were aligned against the sequence representative of

Xanthomonas perforans, strain 91-118, to calculate the SNV profiles using MUMmer algorithm (Kurtz et al. 2004), and ambiguous mappings in alignments were discarded. The trimmed reads were then aligned against the Bowtie database to create sorted and indexed BAM file which were used by the program StrainEst to predict the strain abundance (Figure 2-4) (Albanese and Donati 2017).

The tomato samples collected from field F had *X. perforans* as the only species. Two samples; Tom F F1(17) and Tom F F2 (17) collected in the year 2017 from different plots within the same field had difference within the *X. perforans* population where sample Tom F F1(17) showed the presence of only one SC of *X. perforans* while the sample Tom F F2(17) showed the abundance 2 different SC within *X. perforans*. The samples collected from the same field in the year 2018 showed the presence of two SC of *X. perforans* in sample Tom F (18) and mixed infection with *X. perforans* and *X. euvesicatoria* in pepper sample Pep F (18) with *X. euvesicatoria* being the dominant pathogen.

The pepper samples Pep C (17) and Pep C (18) from another field C in the year 2017 and 2018 had *X. euvesicatoria* as the only species. The tomato Tom C (18) and weed sample Weed C (18) from the same field in the year 2018 had two distinct SCs of *X. perforans*. The tomato samples (Tom E (17) and Tom E (18)) from another field E in both the year 2017 and 2018 had *X. perforans* as the only species present while we did not find any *Xanthomonas* species in pepper sample Pep E (18) collected from this field as suggested also by taxonomic profiling by mOTU (Figure 2-3). The tomato samples Tom A (18), Tom B (17), Tom M (18) and Tom N (18) collected from different other fields in the year 2017 and 2018 showed the presence of *X. perforans* as the only species dominant in the samples.

In order to predict if multiple sequence clusters of *X. perforans* can co-occur in the same field, we further resolved *X. perforans*. Draft genome sequences of *X. perforans* strains from Alabama were combined with 26 additional genomes available from GenBank to construct a core genome alignment with the program Parsnp (Treangen et al. 2014). The resulting 4.03Mb alignment was then subjected to a Bayesian statistical clustering method (Cheng et al. 2013), which predicted the presence of six distinct populations or sequence clusters within *X. perforans* (Figure 2-5). A representative strain from each SC (Table 2-4) was selected and aligned against the completed reference genome of *X. perforans* strain 91-118 using the MUMmer algorithm (Kurtz et al. 2004) to construct a SNV matrix as described before to predict the abundance using StrainEst pipeline (Figure 2-6) (Albanese and Donati 2017).

From the abundance result, mixed infection of different SC in the samples was observed. Tomato samples Tom F F1(17) collected in the year 2017 from field F had the dominance of single SC 3 while the sample Tom F F2(17) from different plot in the same field had the dominance of two SCs; SC3 and SC6. Sample Tom F (18) in the year 2018 from the same field had the introduction of new SC 5 along with the SC 3 from previous year. The tomato Tom C (18) and weed Weed C (18) samples from field C collected in the year 2018 had abundance of SC 6 and SC 5 in both samples but in different level of abundance. However the sample coverage for weed sample was lower than 10% minimum threshold for StrainEst pipeline due to which we cannot make a solid conclusion on the sequence cluster from the weed sample. Other tomato samples Tom A (18), Tom B (17), Tom M (18), Tom N (18) collected in the year 2017 and 2018 had mixed infection with different sequence clusters whereas tomato samples Tom E (17) and Tom E (18) from field E in the year 2017 and 2018 respectively had the infection with single SC 5 in both the year.

Discussion

BLS pathogen of tomato and pepper has shown a great diversity over time in various parts of the world (Schwartz et al. 2015; Timilsina et al. 2016). Different strains of same species differ significantly in terms of their gene content, single nucleotide polymorphism (SNPs), etc. These differences in different strains within the same species are important for understanding microbial adaptation and evolution (Zhu et al. 2015; Schloissnig et al. 2012; Myers et al. 1993). Although the host range of *X. perforans* was traditionally been considered to be restricted to tomato (Potnis et al. 2015), absence of a single gene (either *avrBsT* or *avrXv3*) cause disease on susceptible pepper plants (Schwartz et al. 2015; Timilsina et al. 2019). Variation of pathogens in strain level has been associated to host range adaptations, intraspecies recombination, drug and antibiotic resistance , pathogenicity, etc. in several human and plant pathogens (Lieberman et al. 2014; Rasko et al. 2011; Myers et al. 1993; Timilsina et al. 2019). Study of population and evolutionary genetics of pathogen population helps to understand the epidemiological dynamics and evolutionary history and identify candidate genes responsible for pathogen virulence and host adaptation so an effective strategy can be designed for informed disease management. In this study, we used a culture dependent genomic approach to study the pathogen population structure of BLS *Xanthomonas* in Alabama. We also did a proof of concept study to see if higher resolution in representing diversity can be achieved using shotgun metagenomics in a given sample.

Comparison of the genomes of BLS *Xanthomonas* from Alabama with all the genomes of *Xanthomonas* from GeneBank suggested the strain collected in Alabama as *X. perforans*. Reconstruction of the core genome of *X. perforans* with 45 *X. perforans* genome downloaded from GeneBank and 8 strains used in this study from Alabama showed the diversity of the pathogen. The phylogenetic classification study showed the presence of novel

sequence cluster in *X. perforans* genomes collected in Alabama which we have designated as SC 5 and SC 6 (Figure 2-1). *X. perforans* strains collected in Australia from tomato (Roach et al. 2018) used in this study forms a novel sequence cluster, here designated as SC 7. Presence of novel sequence cluster in *X. perforans* population from Alabama even with limited number of sampling and sequencing just 8 strains gave a new insight of untapped potential of pathogen diversity. As *X. euvesicatoria* was considered as dominant pathogen of pepper, isolation of *X. perforans* strains from pepper samples in Alabama suggest host range expansion. Isolation of *X. perforans* strains on pepper from Florida field was also reported back in 2010 (Timilsina et al. 2015; Schwartz et al. 2015). Presence of two novel SC of *X. perforans* population in Alabama has been inferred as independent evolution of the pathogen with recombination as well as plasmid transfer from multiple *Xanthomonas* species (Newberry et al. 2019).

In order to understand diversity of BLS *Xanthomonas* in different host and non-host, we did a proof of concept study using culture independent shotgun metagenomics during two growing seasons in different areas of Alabama. The taxonomic profiling of the microbial population using mOTU showed the presence of several microbial communities in the phyllosphere (Figure 2-2). From the taxonomic profiling, *Xanthomonas* was dominant pathogen in all the samples except for a single pepper sample. Majority of the tomato sample showed the presence of pathogenic *Pseudomonas cichorii*. As *Pseudomonas cichorii* is known to be pathogenic to tomato (Timilsina et al. 2017), the result suggest that there could be co-infection with multiple genera at the given time in the host phyllosphere. Most of the sample also showed the presence of nonpathogenic *Pseudomonas* and *X. arboricola*. These nonpathogenic species can survive as a saprophyte and co-exists with pathogenic species in the field (Ganeshan and Manoj Kumar 2005; Cesbron et al. 2015). These strains might play an important role in shaping the population structure of pathogenic species as they serve as a

reservoir of several virulence factors that are shared via HGT, recombination or plasmid transfer events (Goss et al. 2013). Co-infection of multiple genus and presence of several saprophytes within a sample might suggest a mutualistic co-existence where both pathogens receive benefits (Mordecai et al. 2016), or it might lead to the emergence of a new recombinant strains where pathogens shares complementary gene sets leading to large scale epidemics in the future (Friesen et al. 2006).

Taxonomic clustering from mOTU showed the mixed infection with multiple species and cannot distinguished between the closely related *X. perforans* and *X. euvesicatoria* species so we further resolved the *Xanthomonas* sp. complex to subspecies level. The abundance results from StrainEst pipeline showed *X. perforans* as a dominant pathogen in tomato and *X. euvesicatoria* in pepper (Figure 4). One pepper sample showed the presence of *X. perforans* in very low abundance suggesting the co-existence of *X. perforans* and *X. euvesicatoria* and *X. perforans* as an emerging pathogen in pepper. Coexistence of *X. perforans* and *X. euvesicatoria* might be responsible for the population structure of *X. perforans* in Florida which were driven by multiple recombination events originating in *X. euvesicatoria* (Timilsina et al. 2019). A strong host preference was seen on the pepper and tomato samples from field C where we didn't see any *X. euvesicatoria* in the tomato and any *X. perforans* in pepper samples. A single weed sample collected from the same field showed the presence of *Xanthomonas perforans*. Weeds acts as a reservoir of pathogenic *Xanthomonas* (Araújo et al. 2015) and might play an important role in evolution of these bacterial pathogens.

We further used the StrainEst pipeline to describe *Xanthomonas perforans* population and predict if multiple SCs of *X. perforans* can co-exist in a field. The analysis of these strains revealed the presence of one or more sequence cluster of *X. perforans* within a single field (Figure 2-6). Tomato samples collected in the year 2017 from field F had abundance of

different SC among different plots. The grower used the seeds and transplants from different sources which might be responsible for the pathogen diversity between the plots. Tomato sample collected in the year 2018 from the same field showed the introduction of novel SC as well as the presence of SC from the previous season. The reason behind introduction of new SC might be multiple source of seeds and transplants the grower gets every year. Use of contaminated seeds from the previous season could be responsible for continuation of similar SC of previous year. Moreover, the grower doesn't care much for the weed in his field which might be the source of reservoir for the epiphytic pathogen. This result describes the role of failure to follow management practices in shaping the pathogen population diversity in the field. Apart from these, there could be several other factors like recombination and HGT that might have involved in creating the novel variant of pathogen. Tomato and weed samples from field C had mixed infection with two different SCs but in different level of abundance. However due to lower coverage from the weed sample than the required threshold of 10%, we cannot make a solid conclusion on the sequence cluster from weed. The tomato samples collected from different fields in both years also showed the presence of mixed infection with multiple SCs. Presence of multiple lineages occupying similar ecological niches might lead to rapid phenotypic evolution via interlineage recombination and ecological divergence in the pathogenic population (Laland et al. 1999).

Overall, the pathogen population structure of BLS *Xanthomonas* in Alabama is diverse with two novel sequence cluster within *X. perforans* population. The proof of concept study using shotgun metagenomics provide a higher resolution of diversity up to SC level and provide the insight of mixed infection with multiple genus and lineages within the given sample. Metagenomic analysis can now pinpoint the presence and abundance of several microbial communities and strains within and between samples. Importantly, this level of resolution helps in checking the presence of several microbial communities in terms of

abundance and distributions which can be missed or misinterpreted based on culturable method only. Moreover, In the future we would like to use such studies to study spatio, temporal dynamics of different genotypes and associated virulence factors in the field and finding truly conserved factors among the diverse pathogens to target breeding efforts.

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Table 2-1: Collection information and assembly statistics, for the *X. perforans* strains sequenced in this study from Alabama.

Strains	**Host**	**County**	**Contigs (N)**	**N50 (kb)**	**Genome size (Mb)**
AL1	Tomato	Lee	86	1.78	5.02
AL65	Pepper	Lee	58	1.91	5.02
AL66	Pepper	Lee	52	2.49	5.02
AL57	Tomato	Lee	55	2.68	4.97
AL33	Tomato	Tuscaloosa	60	2.27	5.22
AL37	Tomato	Tuscaloosa	98	1.31	5.28
ALS7B	Pepper	Tuscaloosa	65	1.87	5.06
ALS7E	Pepper	Tuscaloosa	88	1.39	5.15

Table 2-2: List of *Xanthomonas perforans* strains used in phylogenetic analysis

***X. perforans* strains**	**Collection location**	**Host isolated**	**Year isolated**	**Reference**
X. perforans strains from United States				
Xp 4B	Citra, FL	Tomato	1998	(Schwartz et al. 2015)
Xp 2010	Hendry County, FL	Pepper	2010	(Schwartz et al. 2015)
Xp TB6	Hillsborough, FL	Tomato	2013	(Schwartz et al. 2015)
Xp 3-15	Decatur Co., GA	Tomato	2006	(Schwartz et al. 2015)
Xp 4-20	Decatur Co., GA	Tomato	2006	(Schwartz et al. 2015)
Xp 5-6	Decatur Co., GA	Tomato	2006	(Schwartz et al. 2015)
Xp 7-12	Manatee Co., FL	Tomato	2006	(Schwartz et al. 2015)
Xp 8-16	Manatee Co., FL	Tomato	2006	(Schwartz et al. 2015)
Xp 9-5	Manatee Co., FL	Tomato	2006	(Schwartz et al. 2015)
Xp 10-13	Manatee Co., FL	Tomato	2006	(Schwartz et al. 2015)
Xp 11-2	Palm Beach Co, FL	Tomato	2006	(Schwartz et al. 2015)
Xp 15-11	Miami-Dade Co., FL	Tomato	2006	(Schwartz et al. 2015)
Xp 17-12	Collier Co., FL	Tomato	2006	(Schwartz et al. 2015)
Xp 18-15	Collier Co., FL	Tomato	2006	(Schwartz et al. 2015)
Xp GEV839	Hardee Co., FL	Tomato	2012	(Schwartz et al. 2015)
Xp GEV872	Immokalee, FL	Tomato	2012	(Schwartz et al. 2015)
Xp GEV904	Hillsborough, FL	Tomato	2012	(Schwartz et al. 2015)
Xp GEV909	Collier Co.	Tomato	2012	(Schwartz et al. 2015)
Xp GEV915	Hillsborough, FL	Tomato	2012	(Schwartz et al. 2015)

***X. perforans* strains**	**Collection location**	**Host isolated**	**Year isolated**	**Reference**
Xp GEV917	Hillsborough, FL	Tomato	2012	(Schwartz et al. 2015)
Xp GEV936	Lee, FL	Tomato	2012	(Schwartz et al. 2015)
Xp GEV940	GCREC, FL	Tomato	2012	(Schwartz et al. 2015)
Xp GEV968	Manatee Co., FL	Tomato	2012	(Schwartz et al. 2015)
Xp GEV993	Hendry Co., FL	Tomato	2012	(Schwartz et al. 2015)
Xp GEV1001	Quincy, FL	Tomato	2012	(Schwartz et al. 2015)
Xp GEV1026	West Coast, FL	Tomato	2012	(Schwartz et al. 2015)
Xp GEV1044	Collier Co., FL	Tomato	2012	(Schwartz et al. 2015)
Xp GEV1054	Manatee Co., FL	Tomato	2012	(Schwartz et al. 2015)
Xp GEV1063	Collier Co., FL	Tomato	2012	(Schwartz et al. 2015)
Xp 91-118		Tomato	1991	(Potnis et al. 2011)
Xp LH3		Tomato	2010	(Richard et al. 2017)
Xp GEV2120	Collier Co.	Tomato	2016	(Abrahamian et al. 2019)
X. perforans strains from Australia				
Xp BRIP62383	Bowen	Tomato	2012	(Roach et al. 2018)
Xp BRIP2398	Bowen	Tomato	2014	(Roach et al. 2018)
Xp BRIP62404	Bowen	Tomato	2014	(Roach et al. 2018)
Xp BRIP62397	Brisbane	Tomato	2014	(Roach et al. 2018)
Xp BRIP62386	Bundaberg	Tomato	2012	(Roach et al. 2018)
Xp BRIP62405	Bundaberg	Tomato	2014	(Roach et al. 2018)
Xp BRIP63262	Bundaberg	Tomato	2015	(Roach et al. 2018)

***X. perforans* strains**	**Collection location**	**Host isolated**	**Year isolated**	**Reference**
Xp BRIP63565	Bundaberg	Tomato	2015	(Roach et al. 2018)
Xp BRIP63666	Bundaberg	Tomato	2015	(Roach et al. 2018)
Xp BRIP62389	South Turramurra	Tomato	2013	(Roach et al. 2018)

Table 2-3: Collection information of samples used in metagenomics study from Alabama

Samples	Host	County	Location	Year collected
Tom C (18)	Tomato	Lee	Community garden	2018
Tom E (18)	Tomato	Macon	E.V. Smith Research Station	2018
Tom N (18)	Tomato	Etowah	Commercial farm	2018
Pep E (18)	Pepper	Macon	E.V. Smith Research Station	2018
Pep C (18)	Pepper	Lee	Community garden	2018
Weed C (18)	Weed	Lee	Community garden	2018
Tom A (18)	Tomato	DeKalb	Commercial farm	2018
Tom F (18)	Tomato	Tuscaloosa	Foster	2018
Pep F (18)	Pepper	Tuscaloosa	Foster	2018
TM18	Tomato	Macon	Commercial farm	2018
Pep C (17)	Pepper	Lee	Community garden	2017
Tom E (17)	Tomato	Macon	E.V. Smith Research Station	2017
Tom F F1 (17)	Tomato	Tuscaloosa	Grower's field	2017
Tom F F2 (17)	Tomato	Tuscaloosa	Grower's field	2017
Tom B (18)	Tomato	Baldwin	Commercial farm	2018

Table 2-4: Representative strains used to create SNV matrix

X. perforans **and** ***X. euvesicatoria*** **species complex**	***X. perforans*** **species**
Xp GEV993	*Xp* AL 66
Xeu 85-10	*Xp* AL33
Xp 2010	*Xp* AL 57
Xeu F1	*Xp* LH3
	Xp GEV 993
	Xp 2010

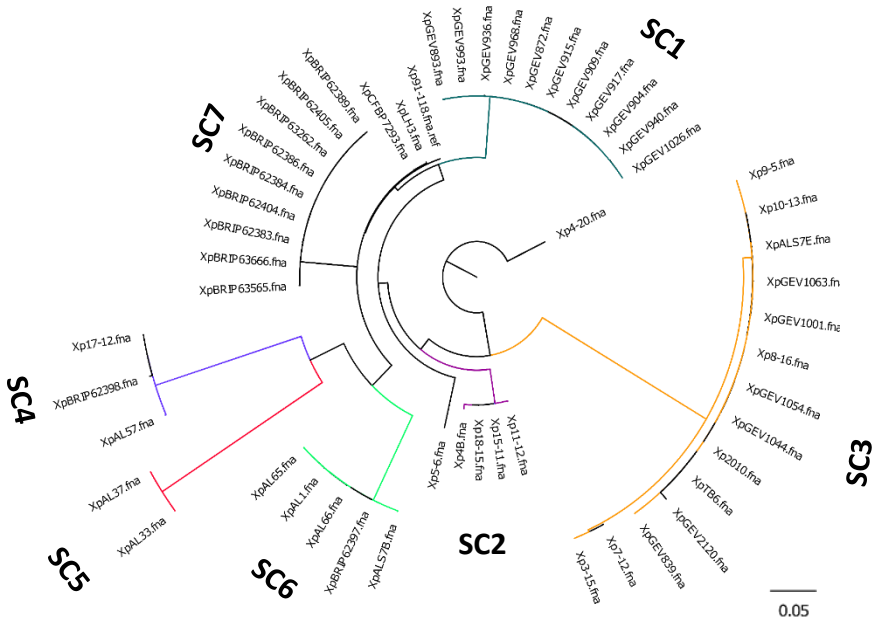

Figure 2-1: Midpoint rooted maximum likelihood phylogeny of *Xanthomonas perforans* strains isolated from different host. Different color branches represent different sequence cluster identified in the first level of HierBAPS hierarchy.

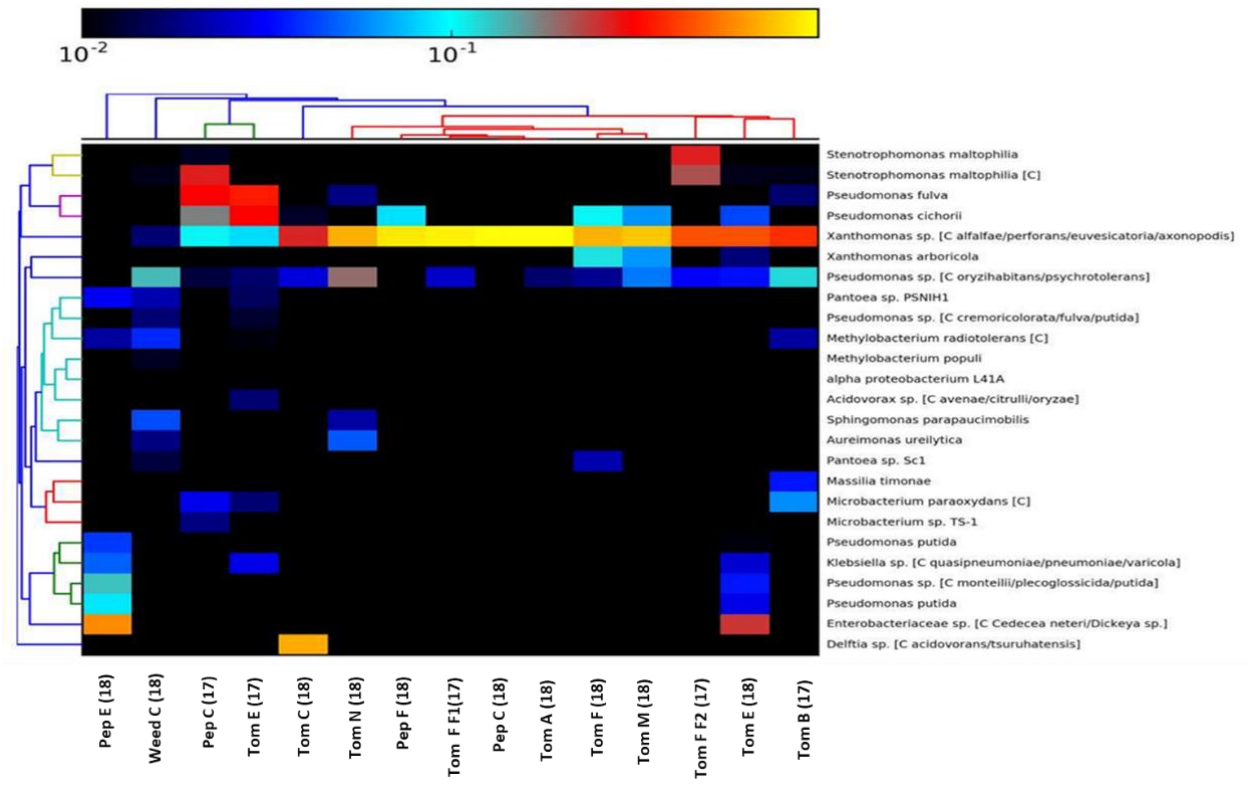

Figure 2-2: Heatmap of taxonomic classification (rows) of microbial species in different samples. Color intensities represent the abundance of the microbial species in the sample with yellow being highly abundance and blue being low abundant. Different columns represent different samples where Tom represents tomato, Pep represents pepper, letters E, C, F, N, M, A, N, and B represents different fields the sample collected from and the number in the parenthesis represents the year the sample collected from.

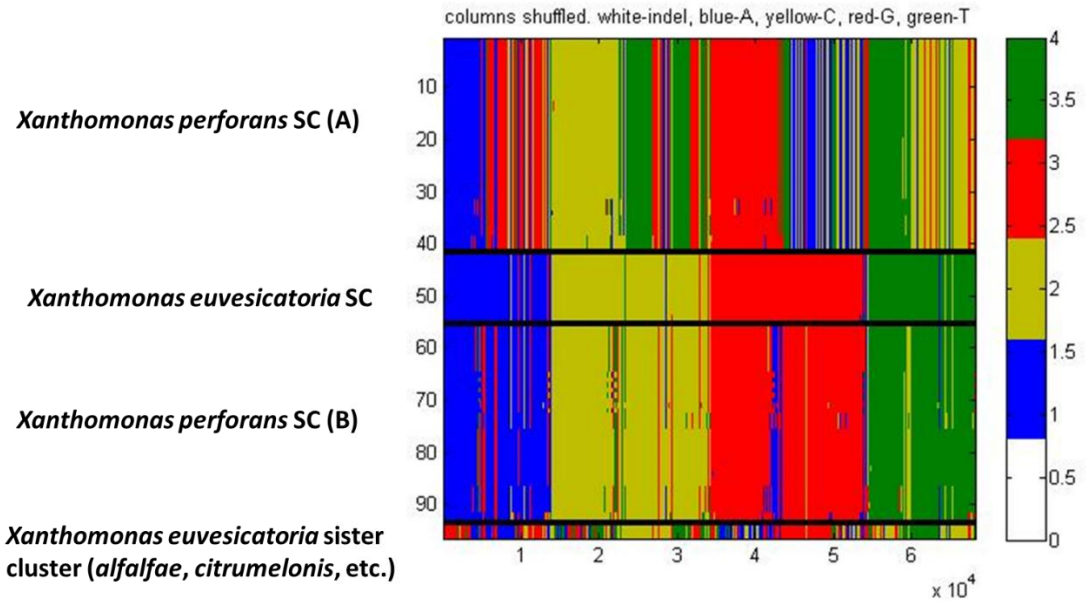

Figure 2-3: Hierarchical Bayesian Analysis of Population Structure (hierBAPS) of *Xanthomonas perforans*, *Xanthomonas euvesicatoria* and its sister clade which includes *X. alfalfae*, *X. citrumelonis* and related species shows the presence of four subpopulations. Green, T; blue, A; red, G; yellow, C.

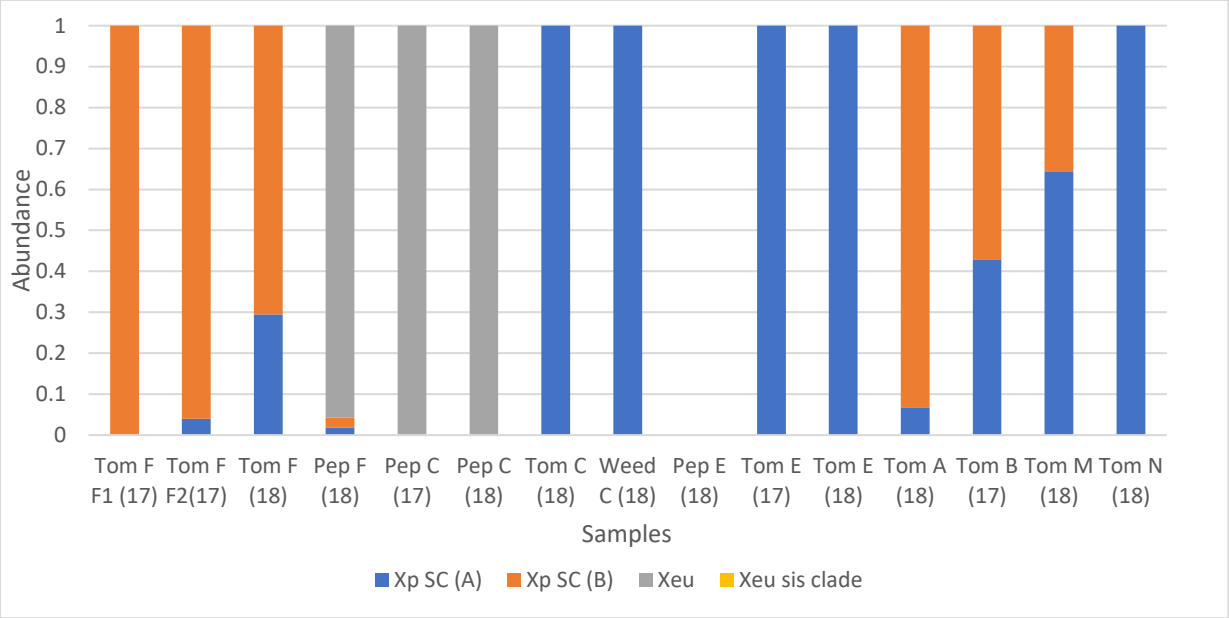

Figure 2-4: Stack bar plot showing abundance of *Xanthomonas perforans* and *Xanthomonas euvesicatoria* against different samples as predicted by StrainEST pipeline. Sample from tomato, pepper and weed are in the horizontal axis where Tom represents tomato, Pep represents pepper, letters E, C, F, N, M, A, N, and B represents different fields the samples were collected from and the number in the parenthesis represents the year the sample was collected.

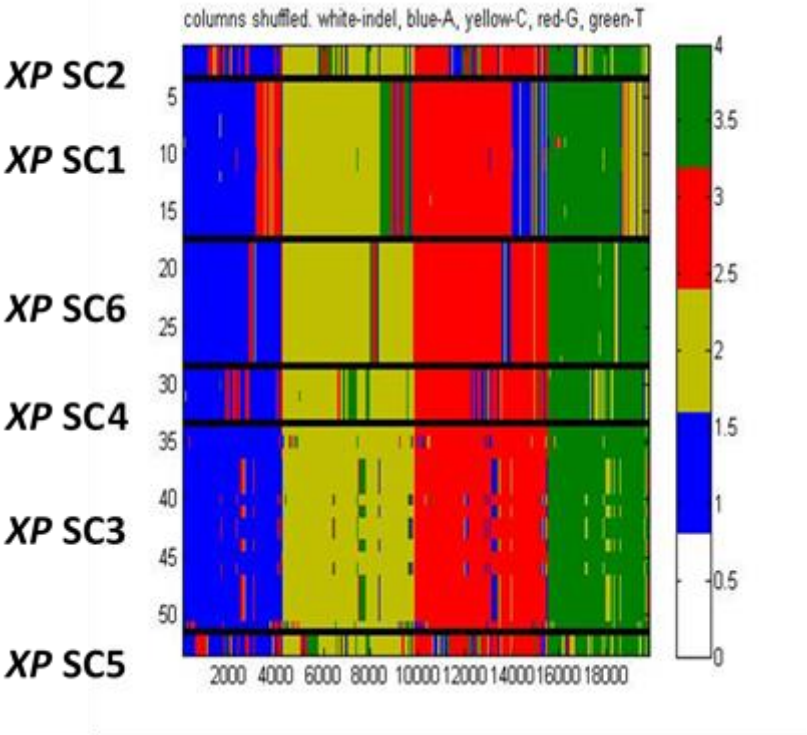

Figure 2-5: Hierarchical Bayesian Analysis of Population Structure (hierBAPS) of *Xanthomonas perforans* shows the presence of six subpopulations. Green, T; blue, A; red, G; yellow, C.

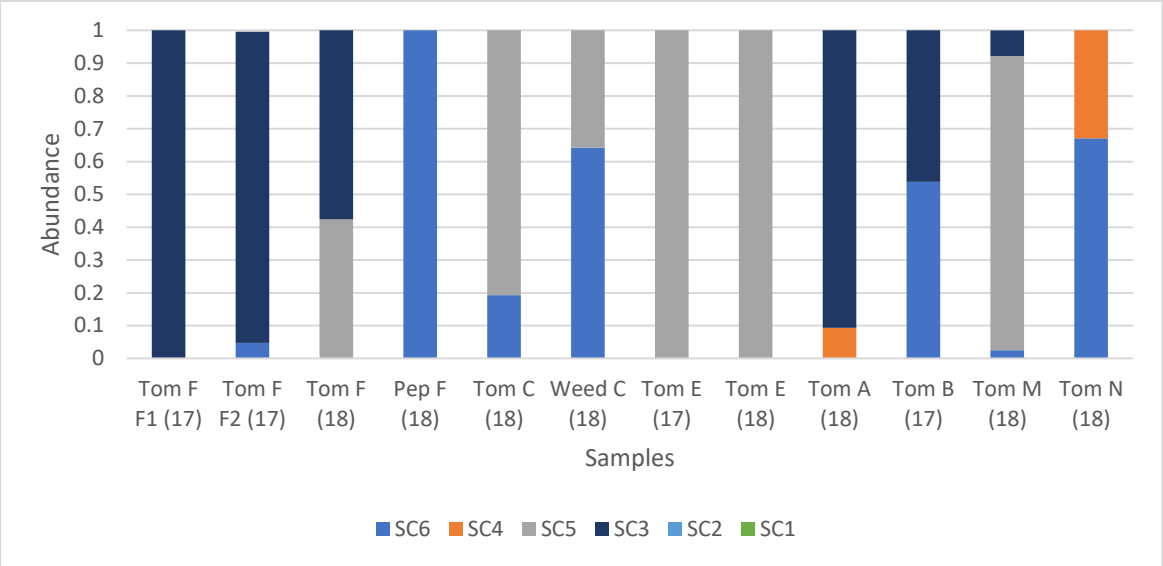

Figure 2-6: Stack bar plot showing abundance of *Xanthomonas perforans* sequence clusters in different samples as predicted by StrainEST pipeline. Sample from tomato, pepper and weed are in the horizontal axis where Tom represents tomato, Pep represents pepper, Different letters E, C, F, N, M, A, N, and B represents different fields the samples were collected from and the number in the parenthesis represents the year the sample collected

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3. CHAPTER THREE

Characterization of chromosomally encoded copper resistance in *Xanthomonas perforans* and investigate its transfer potential under *in-vitro* and *in planta* conditions

Abstract

Copper bactericides have been used since decades for the control of bacterial plant pathogens. Repeated use of copper bactericides against bacterial spot (BLS) xanthomonads has led to spread of copper resistance trait in the pathogen population. Transfer of copper resistance genes via large conjugative plasmid has been a well characterized mechanism in spread of copper resistance in BLS pathogens. We recently identified copper-tolerant *Xanthomonas perforans* strains that contained chromosomally-encoded copper resistance genes on a genomic island. Interestingly, the structure of this island differs from the previously reported chromosomal island of *X. vesicatoria* strain XVP26. In this study, we characterized the genetic organization of this island and investigated the transfer potential of chromosomally-encoded copper resistance genes to the copper sensitive strains under *in-vitro* and *in planta* conditions. Both *in vitro* and *in planta* experiments failed to obtain transductants/transconjugants, indicating that either experimental conditions used in this study were not conducive for the transfer or such transfer frequency is very low.

Introduction

Copper is an essential element for growth and development of both prokaryotic and eukaryotic organisms. It is an essential co-factor for a number of redox reactions mostly involved in respiration, melanin formation, uptake and transport of iron, superoxide

detoxification, etc. (Garcia-Horsmant et al. 1994; Cooksey 1994; Samanovic et al. 2012). Despite being an essential enzymatic co-factor, copper above a certain threshold often results in severe and irreversible cellular damage (Li et al. 2019; Ladomersky and Petris 2015). Various aerobic and anaerobic mechanism for copper toxicity has been described in plant pathogenic bacteria (Freinbichler et al. 2011; Yoshida et al. 1993; Liochev and Fridovich 2002). (Macomber and Imlay 2009; Hiniker et al. 2005). In recent decades, excessive use of copper-based compounds in agricultural systems have resulted in evolution of detoxification systems in phytopathogenic bacteria that protect themselves from toxic concentration of copper (Adaskaveg 1985; Bender and Cooksey 1986b; Ritchie and Dittapongpitch 1991; Sundin 1989). Intra and extracellular sequestration, enzymatic detoxification, and active efflux helps to maintain copper resistance (Cu^R) in bacterial cells (Bondarczuk and Piotrowska-Seget 2013). Recent studies has shown that a highly regulated system of transcriptional regulators, soluble chaperones, membrane transporters, and target cuproproteins found in various bacterial community are responsible for copper homeostasis that ensures the prevention from toxic effects (Argüello et al. 2013).

Genes responsible for imparting copper homeostasis as well as resistance or tolerance to the high levels of copper compounds have been identified and characterized in various pathosystem (Ladomersky and Petris 2015). (Behlau et al. 2011) defined several *cop* and *coh* genes responsible for Cu^R and homeostasis in various *Xanthomonas* sp. When copper concentration exceeds the particular level, most gram positive and negative bacteria synthesize P-type copper export ATPases, resistance-nodulation cell division (RND) and multicopper oxidases type efflux system as principal defense determinants to cope with excess copper (Rademacher and Masepohl 2012). The genes conferring Cu^R in bacteria are often present in plasmids and organized in an operon (Lim and Cooksey 1993; Dupont et al. 2011). Cu^R is encoded by the *cop* genes (*copA*, *copB*, *copC* and *copD*) in several strains of

Cupriavidus, *Pseudomonas*, and *Xanthomonas* and by the *pco* genes (*pcoA*, *pcoB*, *pcoC* and *pcoD*) in *Escherichia coli* (Mellano and Cooksey 1988; Voloudakis et al. 2005; Monchy et al. 2007; Tetaz and Luke 1983).

With the overuse of copper-based compounds in the modern agricultural systems, spread of Cu^R /tolerance genes in microbial communities associated with plants has been a concern dating back to 1960s (Stall et al. 1986; Minsavage 1990b). Several modes of such spread of Cu^R genes have been described in plant pathogenic bacteria. Apart from vertical transmission of copper/heavy metal resistance, genes under continuous selection pressure, horizontal gene transfer (HGT) events mediated by plasmids encoding heavy metal resistance genes have been primary mechanism of the spread within and between species of the bacteria (Tettelin et al. 2005; Vos et al. 2015; Chaguza et al. 2015; Didelot and Maiden 2010; Hanage 2016; Shapiro 2016). Advancement in the genome sequencing technology has helped to understand these HGT events among bacterial populations and to explain spread of heavy metal resistance (Yahara et al. 2016; Driebe et al. 2015; Midha et al. 2017; Huang et al. 2015). Although rare, presence of Cu^R genes on chromosome has been demonstrated recently, where Cu^R genes along with other heavy metal tolerance genes have been identified as cargo genes on genomic islands, also referred to as integrative and conjugative elements (ICE) (Johnson and Grossman 2015). ICEs are the self-transmissible mobile genetic elements (MGE) that are integrated at a specific site (*attB* site) of chromosome, usually a tRNA gene, and flanked by specific direct repeat sequences that define an attachment site on the right (*attR*) and left (*attL*) ends (Zamarro et al. 2016). These ICEs are integrated in the chromosome and encode machinery for conjugation as well as intricate regulatory systems for controlling their excision from the chromosome and their conjugative transfer (Burrus and Waldor 2004). The cargo genes in ICE play an important role in shaping the bacterial behavior (Wozniak and Waldor 2010). In particular, all ICEs contain three distinct modules

that mediate their integration and excision, conjugation, and regulation set of core genes that encode for its excision, circularization, conjugative transfer and site-specific integration in a new host. Apart from heavy metal resistance genes, other cargo genes of ICEs include factors involved in virulence, resistance to antimicrobial compounds, and niche adaptation (Bie et al. 2017; Zhou et al. 2017; Roy Chowdhury et al. 2016).

Emergence of sreptomycin resistant bacterial leaf spot (BLS) *Xanthomonas* in 1960 resulted in the use of copper bactericides as a primary control strategy for the disease (Marco and Stall 1983). However the increase in the pathogenic BLS *Xanthomonas* resistant to copper has poised a great challenge in their management (Obradovic and Jones 2004; Potnis et al. 2015). In BLS *Xanthomonas*, spread of Cu^R has been attributed to a large 200-300 kb self-transmissible plasmid carrying characteristic *cop* operon genes (Canteros et al. 1995; Behlau et al. 2017). Frequent transfer of these plasmids among the strains in the field helps in the transfer of several virulence factors and antibiotic resistance genes to the susceptible strains (Canteros et al. 1995). There has been only one exception, where chromosomal Cu^R was identified in *X. vesicatoria* strain XVP26 isolated in Taiwan (Basim et al. 2005) .

We recently identified several copper-tolerant *X. perforans* (*Xp*) strains collected between 1990-2016 that do not contain these large plasmids. Analysis of complete genomes of representative strains obtained by PacBio SMRT sequencing revealed the integration of Cu^R operon encoding *copL, copA, copB, and copF* on the chromosome mediated by a genomic island. These genomic islands are different from the genomic island of previously reported strain XVP26. These findings were surprising since plasmid-mediated transfer of Cu^R genes has been assumed to be responsible for the emergence of copper resistant strains in BLS *Xanthomonas*. In this chapter, we studied the structure of this genomic island in *Xanthomonas perforans* and confirmed the presence of chromosomal Cu^R using plasmid profiles. We further investigated the transfer potential of chromosomally encoded Cu^R genes

to the copper-sensitive strains via horizontal gene transfer by various *in vitro* and *in planta* experiments.

Methods

Bacteria sample collection, genome sequencing and plasmid extraction

PacBio reads of *Xp* 2010 and *Xp* GEV 904 that were sequenced already were assembled using long read assembler Canu v1.8 (https://github.com/marbl/canu.git). Bacterial leaf spot sample from various field collected from infected tomato in the year 2015 and 2016 in University of Florida was used for the study of chromosomal Cu^R dynamics in *Xanthomonas* population (Table 3-1). Extraction of plasmids was performed as described by (Kado and Liu 1981) with some modifications. Plasmid DNA was subjected to electrophoresis in a 0.5% agarose gel stained precast with GelRed™ (Biotium) according to the manufacturer's protocol (Crisafuli et al. 2015) with Tris-Acetate buffer (2.2 V/cm for 4 h). Gels were imaged using an ImageQuant LAS 4000 (GE Healthcare) imager with a UV transilluminator and EtBr filter.

Comparison of Cu^R genes among different strains and genome alignment

Basic local alignment search tool for nucleotides (BLASTN) was used for the comparison of nucleotide sequences of clones carrying Cu^R genes between *Xp* 2010, *Xp* LH3 and previously sequenced *Stenotrophomonas maltophilia* strain *Stm* K279a (Crossman et al. 2008), *Xp* 7782 (Voloudakis et al. 2005) and Xac, *Xanthomonas alfalfae* subsp. *citrumelonis* strain *Xac* 1381 (Behlau et al. 2013) in National Center for Biotechnology Information (NCBI) (Johnson et al. 2008). Genome comparison to enable visualization of conserved and variable regions among the strain was performed using program Mauve with default settings (Darling et al. 2004).

Assessing the level of CuR among plasmid and chromosomal CuR strains.

Different chromosomal and plasmid borne Cu^R strains (Table 3-1) were selected based on the plasmid profile and growth on nutrient agar plate amended with 200 ppm of copper to assess the level of Cu^R. The strains were grown overnight on nutrient agar amended with 20 ppm copper for the induction of Cu^R (Basim et al. 2005). The growth was then suspended in 0.01M $MgSO_4$ and dilution plated (10^2, 10^3 and 10^4 colony forming unit (CFU)/mL) in copper plate amended with 100, 200, 250, 300, 350 and 400 ppm of copper. Copper was added to the medium as copper sulfate heptahydrate ($CuSO_4$. $7H_2O$) from a 50 mg/mL stock solution. Plates were incubated at 28°C for 48 h before assessing the growth. The experiment was performed once with three replications. Cu^R *X. euvesicatoria* (*Xeu*) strain *Xeu* 75-3 and copper sensitive strain *Xeu* 85-10 was used as positive and negative controls, respectively.

Selection of recipient strains and in planta studies

The donor and recipient strains for the co-infiltration experiment (Table 3-1) were distinguished by assessing the growth of different strains on nutrient agar medium amended with 200, 250 and 300 ppm of copper. Before challenging the strains into different concentration of copper, the strains were grown overnight in nutrient agar plates with 20 ppm of copper for induction of Cu^R (Basim et al. 2005). The strains were then suspended in 0.01M $MgSO_4$ and adjusted to approximately 3 x 10^8 CFU/mL using a spectrophotometer (absorbance 0.3 at 600 nm). Aliquots of 5 µL of the suspension were then spotted in triplicate in nutrient agar plate amended with 200, 250 and 300 ppm of copper. The plates were incubated at 28°C for 48 hours to assess the growth. *Xp* 2010 and *Xp* 91-118 strains were used as positive and negative controls, respectively. Streptomycin resistant mutants of recipient strains (Table 3-1) were obtained by dilution plating of the overnight grown culture on

nutrient agar amended with streptomycin (50 mg/mL) and selecting spontaneous mutants growing on the plates.

Recipient (marked with antibiotic marker streptomycin) and donor strains were grown overnight at 28°C on nutrient agar containing appropriate antibiotic marker. The bacterial growth was then suspended in 0.01 M $MgSO_4$ and adjusted to approximately 3 x 10^8 CFU/mL using a spectrophotometer (absorbance 0.3 at 600 nm). Equal volume of donor and recipient strains was infiltrated into the mesophyll tissue of FL 8000 cultivar of tomato using a 1 ml hypodermic sterile syringe (BD Biosciences). The inoculated plants were kept in growth chamber with a day temperature of 28°C and night temperature of 25°C and were sampled in every 24 and 48 hours. About 0.78 cm^2 of the inoculated leaf tissue were removed from each leaflet with a sterile cork borer and homogenized with the help of Dremel tool (Dremel, PA) in 1 ml Eppendorf tube with 0.01 M $MgSO_4$ and plated for growth in nutrient agar plate with selective antibiotic marker streptomycin and copper (250 ppm). The plates were kept in an incubator at 28°C for 48 hours. The experiment was repeated three times with three replications.

For dip inoculation experiment, the donor and recipient strains used in the study were grown overnight in nutrient agar amended with selective antibiotics. The growth then was suspended in 0.01 M $MgSO_4$ and adjusted to approximately 3 x 10^8 CFU/mL using a spectrophotometer (absorbance 0.3 at 600 nm). Inoculations were performed by dipping FL 8000 cultivar of tomato in 1:1 ratio of donor and recipient strains (10^8 CFU/mL), amended with 0.025% (vol/vol) Silwet 77 (Fisher Scientific) for 30 seconds. The plants were then kept in high humidity chamber (>95% humidity) and transferred to the green house after 2 days. The plants were treated with Agriphage solution (Agriphage company) at 1.24 mL/liter of water and copper (Kocide 3000) (1.05 mg/mL) to provide the selection pressure. The plants were treated with phage at 3rd, 8th and 12th day with Kocide on 4^{th,} 9th and 13th day of

inoculation. Then sampling was done at 7, 15 days post inoculation. The experiment was done twice with three replications.

To assess the ability of in planta transfer of chromosomal Cu^R, the leaves were collected in a Ziploc bag (Ziploc®) with 50 ml 0.1% wash buffer (0.05M PBS, 8.5 g NaCl and 0.2ml tween 20 per liter of water). The samples were then sonicated to disturb and remove the bacterial cells from the leaf surface for 20 minutes using sonicator (VWR) and kept in the orbital shaker at 28°C and 200 rpm for 20 minutes for further removal of bacterial cells form the leaf surface. Then the buffer with bacterial cell were removed using pipette into 50 ml tube and centrifuged at 4000 rpm, 4°C for 20 minutes (Eppendorf® 5418 R) to collect all the cells followed by carefully discarding the buffer without disturbing the pellet at the bottom. The cells were transferred to 1.5 ml Eppendorf tubes washed with sterile deionized water and centrifuged at 12000 rpm for 3 minutes followed by discarding of the water without disturbing the pellet. The wash step was repeated once again followed by dissolving all the cells in 1 mL of 0.01 mM $MgSO_4$ solution. Standard 10-fold dilution plating of the cell suspension was carried out in nutrient agar plate with 250 ppm copper and selective antibiotic marker. The plates were kept in the incubator at 28°C for two days and the colonies grown were used for further screening.

In-vitro transfer potential of Cu^R genes

Bacterial strains and culture methods

Several donor and recipient strains used for this experiment are listed in Table 2. Two donor strains *Xp GEV 904* and *Xp* 2010 stored at -80°C was streaked in a nutrient agar plate and kept in incubation for 24 hours at 28°C. The growth was then resuspended in 0.01 M $MgSO_4$ solution and adjusted to absorbance 0.2 using a spectrophotometer (OD_{600}). 200 µl of bacterial suspension was added to 25 ml nutrient broth in 50 ml tubes and tubes were then

kept in an incubator with shaker at 28°C and 250 rpm for 8 hours till the growth reach mid log phage (OD_{600} =0.3).

Optimizing the time required for phage induction after mitomycin treatment

For chemical induction of phage, a culture of each isolate at an optical density of 0.3 (OD_{600}) was treated with 1 mg/mL freshly constituted mitomycin C (MMC). The control culture was kept unmodified. 200 µl of both MMC treated and untreated bacterial solution was kept in 96 well plate and the optical density was subsequently measured every 30 minutes using Cytation™ 3 plate reader (Biotek) for 15 hours at 28°C and 200 rpm. Increased in the optical density at the beginning followed by sharp decrease in OD in cultures treated with MMC was marked as an indicative of lysis. From the induction assay, the time required for activation of prophage in the culture after addition of MMC was determined.

Transmission electron microscopy

MMC treated culture were passed through 0.2 µm syringe filter (VWR) to obtain the phage lysate. Transmission electron microscopy (TEM) of lysate was performed at the Auburn University Research Instrumentation Facility. Around 8 µl of phage suspension was placed onto a glow-discharged Formvar/carbon 300 mesh copper grid and allowed to adsorb for around 20 minutes. Excess liquid was removed with filter paper followed by a staining with a drop of phosphotungstic acid (PTA). The stain was allowed to sit on the grid for a minute and then removed with the filter paper followed by air drying. The samples were then observed in EM 10 Transmission Electron Microscope (Zeiss) operated at 80kV.

Phage induction and transduction

Cultures were grown to mid-log phase at 28°C with shaking (250 rpm), followed by the addition of MMC (1 mg/mL, Sigma-Aldrich). The mixtures were incubated at 28°C with 250 rpm until complete lysis occur based on the time required for activation of prophage. The lysates were then filter sterilized with syringe filers (0.2 µm, VWR) followed by phage

spot test and the test for antagonism of the donor against the recipient strains as determined by soft agar (0.5%) overlay method (Abedon 2018).

For transduction experiments, the recipient strains were cultured to mid-log phase (3 × 10^8 CFU/mL). The lysate containing approximately 10^8 plaque forming unit (PFU) was added to the lysate and incubated for 30 minutes at 28°C for phage absorption followed by addition of sodium citrate (100 mM, pH 4.5)((Moon et al. 2016). The mixture was centrifuged at 4,000 rpm, 4°C for 15 min (Eppendorf® 5418 R) followed by resuspending the pellet in sodium citrate solution and plated on nutrient agar with 250 ppm copper and supplemented with appropriate antibiotics.

Phage DNA extraction and PCR

The MMC treated culture lysates were used to extract the DNA according to the protocol from Texas A&M Centre for phage technology (Center for Phage Technology, Texas A&M University, College Station 2018) following phage particles precipitation with NaCl (0.5 M final concentration) and polyethylene glycol 8000 (10%, wt/vol), then followed by Phenol chloroform extraction and DNA precipitation. The extracted DNA was used for further analysis.

In-vitro and in planta experiment colonies selection, sequencing, and assembly

Representative transconjugants collected from both *in-vitro* and *in planta* experiment were selected randomly for draft genome sequencing. Genomic DNA was extracted using CTAB-NaCl method as described by (Howland 1996) with some modifications. The degradation and contamination of the genomic DNA was monitored on 0.5% agarose gels and DNA concentration was measured using Qubit® DNA Assay Kit in Qubit® 2.0 Flurometer (Life Technologies, CA, USA) and submitted to the Georgia Genomics and Bioinformatics Core, University of Georgia for library preparation and sequencing. Paired-

end reads generated by Illumina MiSeq Micro (PE150) platform was subjected to adapter filtering, quality trimming and contaminant removal using BBDuk (https://jgi.doe.gov/data-and-tools/bbtools/bb-tools-user-guide/bbduk-guide/). Contigs were assembled with SPAdes version 3.5.0 (Bankevich et al. 2012) with kmers of 127, 117, 107, 97, 87, 77, 67 using the 'careful' flag. Genome statistics including GC content, contig number, N50 and genome length were calculated with QUAST web interface (Gurevich et al. 2013).

Reconstruction of core genome

The complete genomes of the sequenced transconjugants were annotated de novo with Prokka (Seemann 2014) and these annotations were used by Roary ((Page et al. 2015) to produce the pan-genome. The maximum-likelihood phylogenetic trees was constructed using FastTree (Price et al. 2009) and visualized in FigTree (v.1.4.2, http://tree.bio.ed.ac.uk/software/figtree/).

Results

PacBio assembly and plasmid profile of Xp 2010 and Xp GEV 904 reveals the absence of Cu^R plasmid

We assembled the PacBio reads using new version of long read assembler, Canu v1.8 (https://github.com/marbl/canu.git) and obtain the contigs of size 5181593, 55316, and 13407 bp in *Xp* 2010 and contigs size of 5047419, 61267, 49029, 23711 bp in *Xp* GEV 904. To further validate this result, plasmid was extracted from *Xp* 2010 and *Xp* GEV 904. The agarose gel electrophoresis of the plasmid DNA shows the absence of Cu^R plasmids in these strains suggesting the chromosomal integration (Figure 3-1). Comparison of Cu^R determinants in *Xp* 2010 and *Xp* GEV 904 with plasmid borne gene cluster in *Xp* LH3 and *Xp* 7782 strains revealed high homology (≥92%) of nucleotide sequences among these strains for *copLAB* genes (Figure 3-2).

Characterization of chromosomal Cu^R

Next, we investigated the nature of chromosomal integration of Cu^R. Characterization of the flanking genes of Cu^R operon indicated presence of 65 kb ICE related structure. Further analysis to identify structure of this ICE indicated presence of phage-related integrase and att site involved in ICE integration, Tn3 DDE transposases, presence of heavy metal resistance and other phage related genes. These phage related genes included partition genes, coat protein encoding genes, virus replication associated proteins, viral RNA helicase, ATP-dependent endonuclease/ nucleotidyl transferase domain containing protein involved in overcoming lysogenization defect.

Comparing copper plasmid from *Xp* LH3 to chromosomal region of Cu^R from *Xp* 2010 indicated conserved sequence and organization for copper operon and other heavy metal resistance clusters except for absence of phage-related genes in *Xp* LH3 plasmid (Figure 3-2). Plasmid-borne Cu^R operon and other heavy metal gene clusters in strain *Xp* LH3 were flanked by Tn3-family transposon element. Thus, it appears that chromosomal and plasmid borne Cu^R clusters do not differ in their sequence identities for heavy metal resistance genes, but they differ in their mechanisms of transfer across populations.

Both the chromosomal and plasmid borne Cu^R can exist in the same field at the same time.

Xanthomonas strains were collected from the same field that was continuously sprayed with copper (Table 3-1). Plasmid profile of these strains in the presence of *Pantoea stewartii* marker suggested the strain *Xp* GEV1918, *Xp* GEV2004 and *Xp* GEV2048 harbors the characteristic Cu^R plasmids similar to the plasmid size that has been described on strain E3. However, the strains *Xp* GEV1918, *Xp* GEV2013 and *Xp* GEV2113 did not poses the characteristic Cu^R plasmid suggesting the possible chromosomal integration. Plasmid profile

analysis of the strains showed that both the chromosomal and plasmid borne Cu^R strains can co-occur in the same field at a same time (Figure 3-4).

Strains with plasmid-borne Cu^R can tolerate higher concentrations of copper as compared to strains carrying chromosomal Cu^R

From the plasmid profile result, chromosomal Cu^R Strain *Xp* GEV2013 and plasmid borne Cu^R strain *Xp* GEV2048 were selected for population experiment under different concentration of copper (100, 150, 200, 250, 300, 350 and 400 ppm). Copper sensitive negative control strain *Xeu* 85-10 did not grow in copper plate higher than 150 ppm. Strain *Xp* GEV2048 has significantly higher population (1.27 log fold higher) compared to strain *Xp* GEV2013 at copper concentration 350 ppm. Strain *Xp* GEV 2013 did not grow at all on nutrient agar plate at copper concentration 400 ppm while the population of *Xp* GEV 2048 was 3.77 log CFU/ml. The copper tolerance test among chromosomal and plasmid borne Cu^R strains showed that plasmid borne Cu^R strains can tolerate higher rate of copper as compared to that of chromosomal Cu^R (Figure 3-5).

In planta transfer of chromosomal Cu^R

We select the recipient strain (Table 3-1) for transfer experiment based on their ability to grow on the nutrient agar plate amended with different concentration of copper. From the plate spot assay, strain *Xp* AL1, *Xp* AL57, *Xp* AL66, *Xp* AL65 *Xp* 91-118 and *Xp* ALS7B were selected as copper sensitive recipient based on their inability to grow on nutrient agar plate amended with 200 ppm of copper (Figure 3-6).

Preliminary attempts to test the potential of chromosomal Cu^R transfer by mixing copper tolerant strain *Xp* 2010 and *Xp* GEV 904 strains carrying chromosomally encoded Cu^R and copper-sensitive strain *Xp* 91-118 by infiltration in planta were unsuccessful as we failed to get any colonies on a nutrient agar plate amended with 200 ppm copper, indicating

possible different mechanism of chromosomal transfer associated with *Xp* ICE compared to XVP26 ICE (Basim et al. 2005).

Dip inoculation of donor strain *Xp* GEV 904 and *Xp* 2010 with recipient strain *Xp* 91-118 resulted in few colonies in the plate where we selected the representative strains, extracted DNA and performed whole genome sequencing. Polymerase chain reaction (PCR) amplification of the copper resistant genes *copA*, *copL* and *copB* in colonies from *in planta* experiment in presence of donor and recipient strains showed positive amplification for these genes suggesting the presence of the Cu^R genes (Figure 3-7). Analyzing the genomes of these colonies based on core genome phylogeny of single nucleotide polymorphism (SNPs) using Parsnp (Treangen et al. 2014) showed the contaminants in our experiment from donor strain *Xp* GEV 904 as most of the isolates obtained from *in planta* experiment branched with the donor *Xp* GEV 904 in the phylogeny (Figure 3-8). Next, we screened the transconjugant genomes for signature genes that would distinguish between donor and recipient strains. Blast search of translated nucleotide databases using a protein query (tblastn) with avirulence protein *avrBsT* which is present intact in *Xp* GEV 904 but absent in *Xp* 2010 and *Xp* 91-118 in NCBI shows the presence of intact *avrBsT* in our colony from *in planta* transfer experiment suggesting the contamination with *Xp* GEV 904. Blast result for *avrBsT* for the combination of *Xp* 2010 and *Xp* 91-118 also showed the presence of intact *avrBsT* suggesting the contamination of *Xp* GEV 904 in this combination as well. Avirulence protein *avrXv3* was intact in *Xp* 91-118 while has frame shift at 69^{th} amino acid (aa) in *Xp* GEV 904 and absent in *Xp* 2010 (Timilsina et al. 2016; Schwartz et al. 2015). Blast result of these avirulence proteins with *in planta* colonies from the combination of *Xp* 2010 and *Xp* 91-118 also showed similar inactivated form with frame shift in 69^{th} aa similar to that of *Xp* GEV 904 suggesting contamination with *Xp* GEV 904.

Investigating the role of phage genes in transfer of chromosomal Cu^R island

Activation of prophage took 7 hours in *Xp* 2010 and 3 hours in *Xp* GEV 904 after MMC treatment as indicated by the growth curve (Figure 3-9). Plate plaque assay with the phage lysate from *Xp* 2010 and *Xp* GEV 904 on different recipient strain to the test of direct antagonism showed that the strains *Xp* AL66, *Xp* AL67, *Xp* 91-118, *Xp* AL57 produce turbid plaque and these strains can be used as a recipient in the further experiment (Figure 3-10). Transmission electron microscopy (TEM) analysis of the phage lysate in both the samples from *Xp* 2010 and *Xp* GEV 904 showed an elongated filamentous structure resembling Cf1c phage (Figure 3-11) (Kuo et al. 1991). However, the length of the phage was longer than (~870 nm) (Mai-Prochnow et al. 2015). Filamentous phages has an advantages of variable length according to the size of the packaged DNA, because the vPIII subunit can be altered to accommodate different DNA sequences leading to a more flexible structure (Karimi et al. 2016). Phage DNA extracted from the lysate was used for the PCR amplification of Cu^R gene *copA* and *copL* where we got the positive amplification for both genes (Figure 3-12) suggesting the presence of Cu^R genes.

Transduction experiment resulted into the few colonies in plate from the combination of donor *Xp* GEV 904 with recipient strains *Xp* AL1, *Xp* AL57 and *Xp* 91-118. PCR amplification of the colonies from plate resulted in the positive amplification of *copA* and *copL* genes suggesting the presence of Cu^R genes (Figure 3-13). In order to further validate the result, we extracted the DNA and send them for whole genome sequencing. Core genome phylogeny of SNPs from the donor, recipient and strains from transduction experiment using Parsnp (Treangen et al. 2014) showed all the sequenced transductants aligned with the donor strain *Xp* GEV 904 suggesting the donor contamination (Figure 3-14). We further analyzed these genomes by blast search of translated nucleotide databases using a protein query (tblastn) with different virulence effectors that are known to be present in donor and recipient

strains against the colonies from transduction experiment. Blast result of avirulence protein *avrXv3, xopAQ,* and *pthXp1* which were found intact in *Xp* AL57 (Newberry et al. 2019) showed inactivation of *avrXv3* with a frame shift on 69th aa and absence of *xopAQ* and *pthXp1* in donor strain *Xp* GEV 904. The blast search of these avirulence proteins against *in-vitro* colony from the combination from *Xp* GEV 904 and recipient *Xp* AL57 showed an inactivated *avrXv3* with frame shift in 69th aa suggesting the colony being likely *Xp* GEV 904. Blast result of the virulence protein *xopAQ* and *pthXp1* in *in-vitro* colony from this combination didn't showed any identity in the genome suggesting absence of these virulence factors. Similarly, the blast result with avirulence protein *avrHah1* which was intact in *Xp* AL1 and *avrXv3* which had insertion sequence in *Xp* AL1 (Newberry et al. 2019) with donor *Xp* GEV 904 which did not have *avrHah1* and inactivated *avrXv3* showed the absence of *avrHah1* and inactivated *avrXv3* with frame shift in 69th aa suggesting contamination from the donor *Xp* GEV 904.

Discussion

Emergence of streptomycin and copper resistant strains has been a major challenge towards the management of BLS in the field (Marco and Stall 1983; Ritchie and Dittapongpitch 1991). As majority of the antibiotics and heavy metal resistance genes in BLS are encoded on self-transmissible plasmids, transfer of plasmid among the strains in the field is the major contribution factor towards the spread of these resistance (Canteros et al. 1995).

Here we sequenced the copper tolerant *Xanthomonas* strain *Xp* 2010 and *Xp* GEV 904 using long-read PacBio SMRT sequencing technology. The long-read assembly using Canu v1.8 showed the absence of large Cu^R plasmids on these strains. Plasmid profile of these strain further proved that these big Cu^R plasmids are missing in these strains suggesting the integration of Cu^R in chromosome. Cu^R in the genus *Xanthomonas* is mostly governed by *copLAB* gene cluster and is present in many species of *Xanthomona*s around the world

(Behlau et al. 2013). Comparison of Cu^R gene cluster from chromosomal encoded Cu^R in *Xp* 2010 and *Xp* GEV 904 showed Cu^R operon in these strains was identical in organization and sequence identity to already characterized plasmid borne Cu^R from *Xp* LH3 and *Xp* 7782 strain. The analysis further suggested that these Cu^R genes in chromosomal Cu^R strains might have integrated from the plasmid. Comparison of the flanking region of the chromosome from *Xp* 2010 and Cu^R plasmid from *Xp* LH3 showed conserved Cu^R operon between the strains (Figure 3-2). Apart from these, other heavy metal resistance genes encoding for cobalt, cadmium and zinc were also conserved among the strains. The only difference between the flanking regions of the plasmid to that of chromosome are the presence of phage related genes, attachment site and Tn3 DDE transposases. The presence of att site and Tn3 DDE transposases indicated that these might be the integrative and conjugative element and the presence of phage gene suggest that the phage might have a role in the induction of ICE. The prophage encoded in ICE does not encode the complete phage machinery but there are helper phages present in the bacterial chromosome that helps in the induction of the ICE. Phage-inducible chromosomal islands found within the chromosomes of both gram positive and gram-positive bacteria hijack viral machinery from helper phages for their transfer (Fillol-Salom et al. 2018; Penadés and Christie 2015). Screening the genome of previously identified Cu^R strain XVP26 showed the presence 120 kb integrative and conjugative element (ICE) including genes for heavy metal resistance and genes involved in $Cu^{R,}$ transport and regulation. The structural organization as well as sequence of this ICE was like that of 95 kb ICE identified from *X. arboricola* pv. *juglandis* CFBP7179 (Cesbron et al. 2015). Characterization of the flanking region of the Cu^R operon in *Xp* 2010 suggested the 65 kb ICE like structure which was different in structure as compared to that of XVP26. Further study on distribution of Cu^R in the pathogenic population showed both form of chromosomal and plasmid borne Cu^R can co-exist in the same field. This finding is also consistent with the

theory that under selection, beneficial plasmid accessory genes are captured by chromosome and the plasmid is lost (Bergstrom et al. 2000).The test for level of Cu^R among the plasmid and chromosomal Cu^R strains showed plasmid borne Cu^R strain *Xp* GEV 2048 can tolerate higher amount of copper as compared to chromosomal Cu^R strain *Xp* GEV 2013. In order to make a solid conclusion that plasmid borne Cu^R can tolerate higher amount of copper compared to chromosomal Cu^R, population experiments with more strains from chromosomal and plasmid borne Cu^R should be carried out in the future.

ICEs have been most extensively studied with respect to their roles in conferring virulence factors and resistance to antimicrobial compounds in various bacteria (Clawson et al. 2016; León-Sampedro et al. 2016; Zhou et al. 2017; Wozniak and Waldor 2010; Johnson and Grossman 2015; Ryan et al. 2017; Chuzeville et al. 2017), rhizobial root nodulation in *Azorhizobium caulinodans* (Ling et al. 2016) and strains of *Mesorhizobium*, *Azorhizobium*, and *Bradyhizobium* (Servín-Garcidueñas et al. 2016; Lee et al. 2008; Wang et al. 2014; Sullivan et al. 1995; Sullivan and Ronson 1998), biofilm formation in marine *Pseudoalteromona* (Wang et al. 2017), various aerobic and anaerobic catabolic properties (Zamarro et al. 2016), genetic plasticity and adaptability of microorganisms for survival in various ecological niches (Suenaga et al. 2017), etc. During their conjugative transfer, ICE gene expression induces the excision of ICE from the donor chromosome to form a double stranded circularized plasmid. The ICE encoded relaxase nicks one nicks one strand of dsDNA to form a transfer DNA (T-DNA) which gets transferred to the recipient cell through the mating pore, synthesize the complementary strand and integrated into the recipient cell and reintegration DNA of the circularized into the donor chromosome(Johnson and Grossman 2015). Presence of phage-related genes, including integrase and additional genes encoding coat protein, viral replication related proteins, similar to those identified in phage-inducible chromosomal islands (PICIs) of gram-negative bacteria (Fillol-Salom et al.

2018) led us to hypothesize that phage could play role in transfer of this ICE. *Xp* 2010 genome contains a complete set of Cf1c prophage genes on the chromosome, not as a part of ICE. A tight association in presence of Cf1c prophage as well as ICE was found in all strains carrying chromosomally encoded Cu^R based on genome analyses. Interestingly, metagenome samples collected across the state of Alabama also showed presence of Cf1c phage (Potnis et al., unpublished data), indicating contribution of this phage towards horizontal gene pool among pathogen populations. Gram-negative ICEs/ PICIs encode *AlpA*, an activator of PICI cycle whose expression depends on the helper phage (Fillol-Salom et al. 2018) and such helper phage is capable of inducing PICI excision, replication and packaging. We identified a transcriptional regulator, DNA-binding protein, located adjacent to phage integrase in *Xp* 2010 that might function similar to AlpA transcriptional regulator from *E. coli*. Presence of helper prophage in the strains carrying ICE also points us to the hypothesis that helper prophage Cf1c might be responsible for induction of ICE, thus, lysogenic strains carrying ICE will act as donors of adaptive traits.

Transfer of these ICE among the strains by co-inoculation failed suggesting a separate mechanism of chromosomal Cu^R transfer as compared to that of XVP26 (Basim et al. 2005). Dip inoculation of the donor strains *Xp* 2010 and *Xp* GEV 904 and recipient strain *Xp* 91-118 in the presence of selection pressure (copper and Agriphage) resulted into some colonies in plate. However, the whole genome alignment of the transconjugants showed the presence of contamination on our experiment as all the strains from *in planta* experiment branched with the donor parents. The contamination from donor was further proved from the blast result using various virulence traits found exclusively in donor or recipient strains.

For *in-vitro* transduction experiment, the time required for the activation of phage by MMC treatment was determined as 7 hours for *Xp* 2010 and 3 hours for *Xp* GEV 904. The antagonistic phage plaque test produce turbid plaque in several recipient strain similar to that

of Cf1c phage (Kuo et al. 1991). Transmission electron microscopy of the phage lysate showed the elongated filamentous structure like that of Cf1c phage. However, the size of this filamentous structure was longer than that of Cf1c phage. Phage DNA extracted from the lysate gives a positive amplification for Cu^R genes which suggest that these Cu^R cluster is being transported by the phage particles. Transduction experiment with the donor lysate from donor and recipient cells gave some colonies *in-vitro* on nutrient agar plate with copper and selective antibiotic marker. The positive amplification for Cu^R genes indicates the presence of Cu^R genes in these strains. Comparison of the core genome SNPs from these colonies using Parsnp (Treangen et al. 2014) showed all strains grouped together with the donor strain *Xp* GEV 904 indicating the donor contamination. Although the phage lysate from the donor cells were passed through 25mm syringe filter (0.2µm, VWR), there might be some contamination during the transfer that the donor cell might had passed during filtration process. This result shows that the importance of control and sequencing the parents in our experimental design and lack of an appropriate control may lead to misleading interpretation in an experiment.

Continuous use of copper for the control of BLS has resulted in the emergence of Cu^R BLS *Xanthomonas* which creates doubts in sustainability of the production system. Cu^R in *Xanthomonas* was supposed to be mediated by Cu^R plasmids. Recently, we identified some of the *X. perforans* showing copper resistance operon in ICE integrated into the chromosome. Several *in-vitro* and *in planta* experiment performed to check the transfer potential of these Cu^R did not get success suggesting there could be a different mechanism for the transfer of this chromosomal Cu^R, or we were not able to mimic the right condition that was necessary for the transfer. Recent findings of Cu^R in ICE integrated into the chromosome lead us to different questions. As we know that there has been overuse of copper despite presence of Cu^R in BLS, has this overuse resulted in the fixation of Cu^R cluster in the chromosome? Moreover, there is always a cost associated to the pathogen in keeping this large plasmid

where the integration of Cu^R into the chromosome make the pathogen get rid of this. Given the failure of chromosomal transfer of Cu^R, in the future we would like to monitor the abundance of chromosomal/plasmid borne Cu^R in the fields. We would also like to monitor the copper use pattern and its influence on pathogen population in southeastern United States. These studies will further help in understanding the mechanism chromosomal Cu^R transfer and the influence of various selection pressure in these pathogenic population.

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Table 3-1: List of different *Xanthomonas* strains used in the study

Strains	**Location**	**Host**	**Year of isolation**	**References**
Xanthomonas perforans				
Xp 2010	Hendry Co., FL	Pepper	2010	(Schwartz et al. 2015)
Xp GEV 904	Hillsborough Co., FL	Tomato	2012	(Schwartz et al. 2015)
Xp GEV1918	Collier Co., FL	Tomato	2015	(Abrahamian et al. 2019)
Xp GEV2004	Polk Co., FL	Tomato	2015	(Abrahamian et al. 2019)
Xp GEV2013	Manatee Co., FL	Tomato	2015	(Abrahamian et al. 2019)
Xp GEV2048	Polk Co., FL	Tomato	2015	(Abrahamian et al. 2019)
Xp GEV2058	Polk Co., FL	Tomato	2015	(Abrahamian et al. 2019)
Xp GEV2113	Collier Co., FL	Tomato	2016	(Abrahamian et al. 2019)
Xp GEV2119	Collier Co., FL	Tomato	2016	(Abrahamian et al. 2019)
Xp GEV2120	Collier Co., FL	Tomato	2016	(Abrahamian et al. 2019)
Xp 91-118		Tomato	1991	(Potnis et al. 2011)
Xanthomonas euvesicatoria				
Xeu E3			1961	
Strains from Alabama				
Xp Al65	Lee Co., AL	Tomato	2017	(Newberry et al. 2019)
Xp Al66	Lee Co., AL	Pepper	2017	(Newberry et al. 2019)
Xp AL57	Lee Co., AL	Pepper	2017	(Newberry et al. 2019)
Xp AL1	Lee Co., AL	Tomato	2017	(Newberry et al. 2019)
Xp ALS7E	Tuscaloosa Co., AL	Pepper	2018	(Newberry et al. 2019)

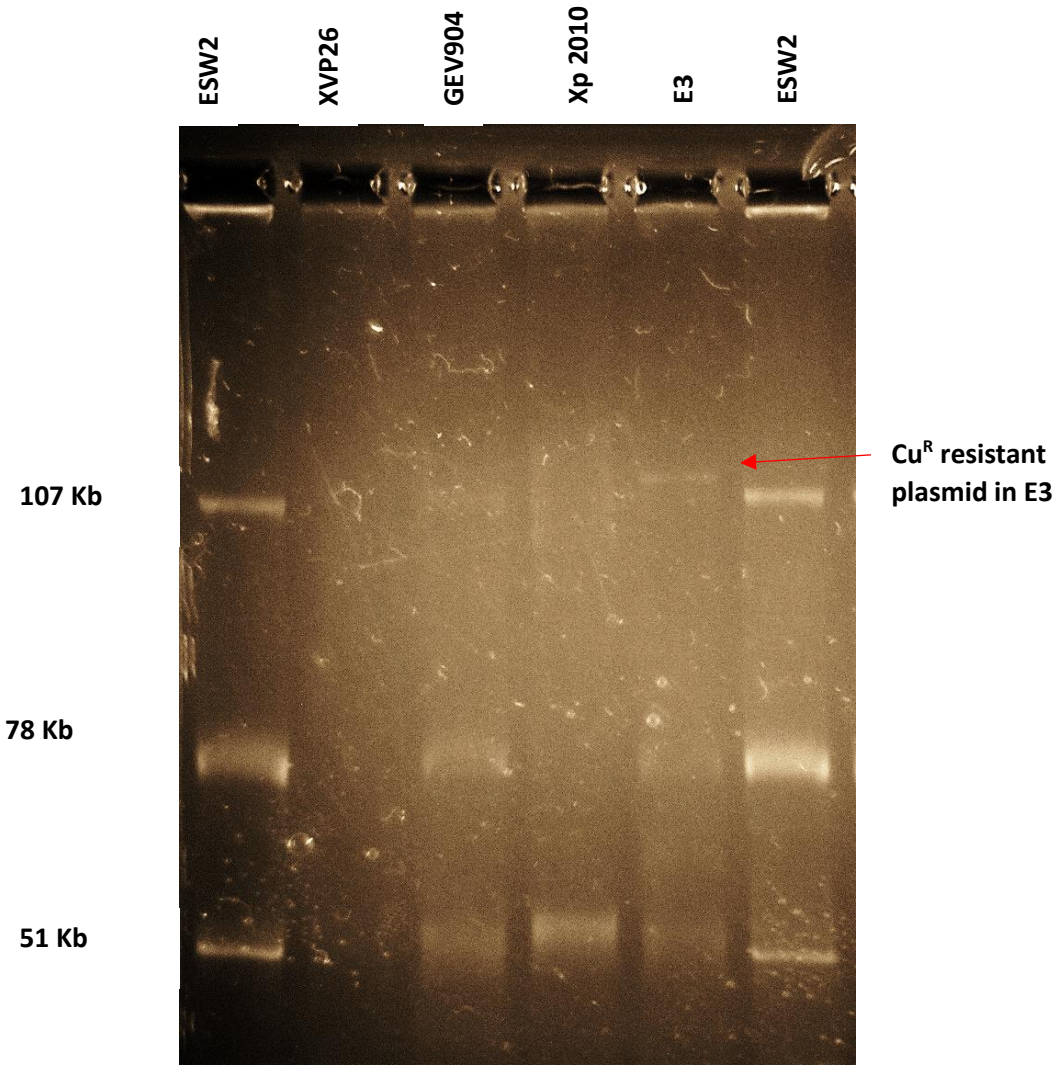

Figure 3-1: Agarose gel electrophoresis of plasmid DNA of different *Xanthomonas* strains. Big copper resistant plasmids are missing in *Xp* 2010 and *Xp* GEV 904. Strain XVP26 is used as a positive control for chromosomal copper resistance. Strain E3 is used as a positive control for copper resistance plasmids. Letters indicate the size class in kb. Plasmid of strain SW2 of *Pantoea stewartii* is used as a marker.

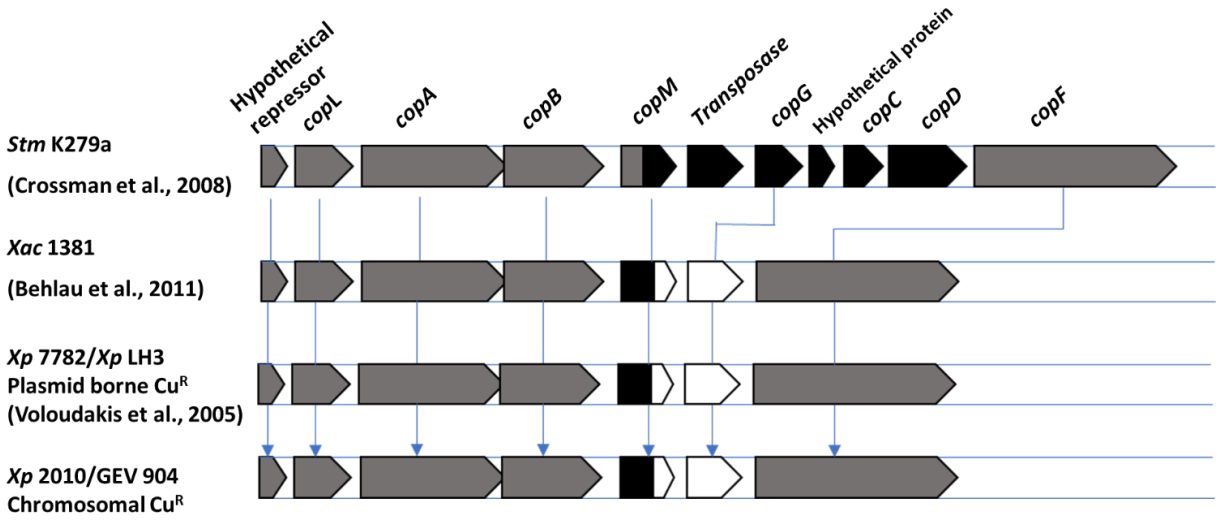

Figure 3-2: Comparison of copper resistance gene cluster among different *Xanthomonas* species showing chromosomal copper resistance and plasmid borne copper resistance operon are identical in sequence identity as well as gene organization.. Areas with the same color indicate conservation of nucleotide sequence among the strains with identity ≥92%. *Stm, Stenotrophomonas maltophilia*; *Xac, Xanthomonas alfalfae* subsp. *citrumelonis*; Xp, *Xanthomonas perforans.*

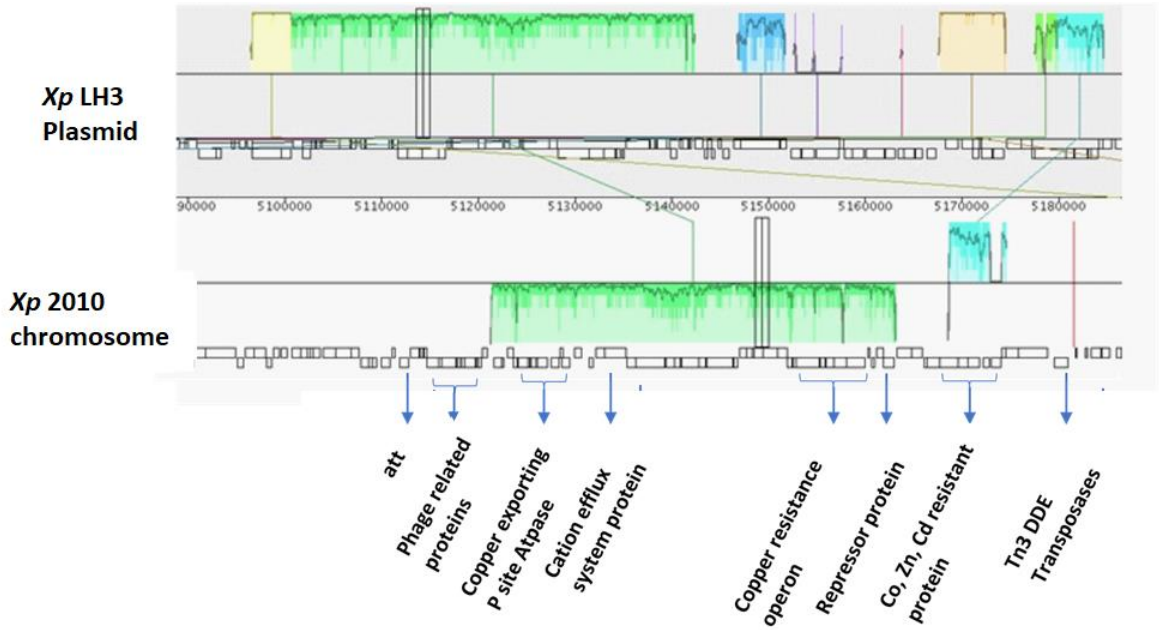

Figure 3-3: Mauve alignment of *Xp* 2010 ICE carrying copper resistance genes with *Xp* LH3 plasmid. Similar color-coded regions showing the conserved regions between the chromosome and plasmid. Copper and heavy metal resistance operons are identical on plasmid and chromosomal ICE. Phage related proteins, att site and Tn3 DDE transposases found in chromosomal ICE was absent in plasmid.

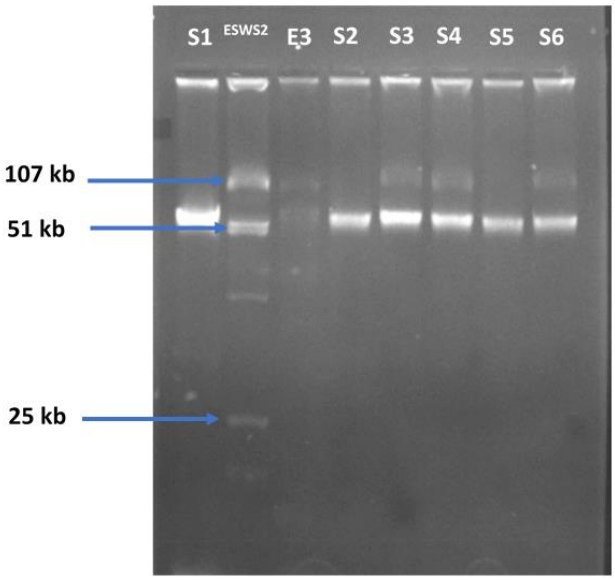

Figure 3-4: Agarose gel electrophoresis of plasmid DNA from different *Xanthomonas* strains collected from same field showing the presence of chromosomal (strains S1, S2 and S5) and plasmid borne copper resistance (strains S3, S4 and S6). Strain E3 is used as a positive control for strain having the copper resistance plasmid. Plasmid of strain SW2 of *Pantoea stewartii* is used as a marker.

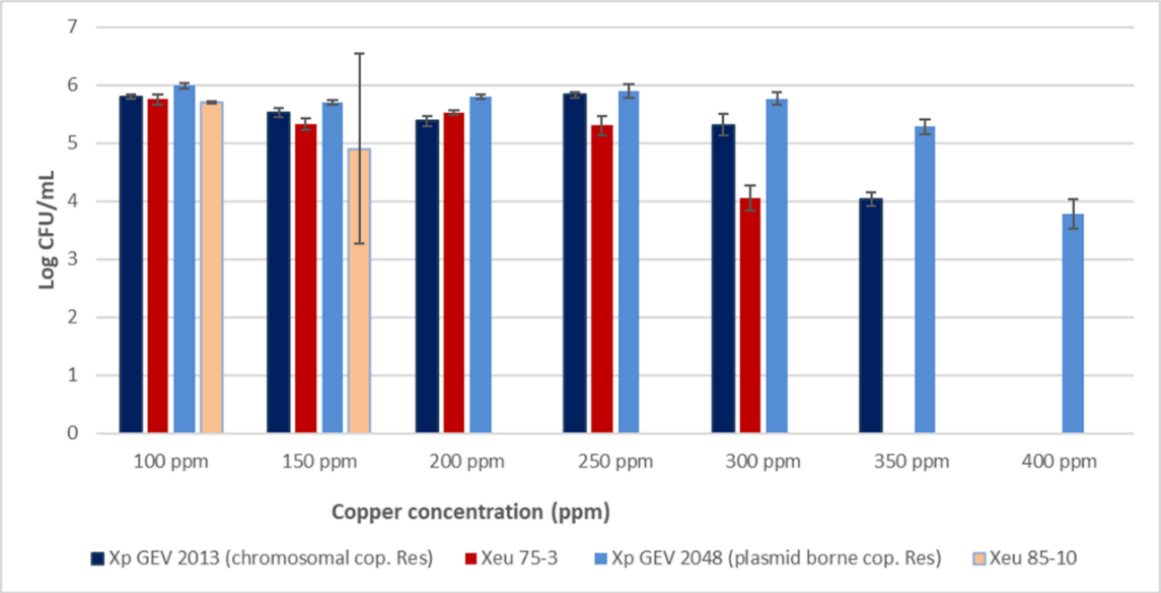

Figure 3-5: Growth of different *Xanthomonas* strains with plasmid and chromosomal copper resistance in nutrient agar amended with copper (100, 150, 200, 250,350 and 400 ppm). Strain *Xeu* 85-10 is used as a negative control and *Xp* 75-3 as a positive control. The strain *Xp* GEV2048 has copper resistant plasmid while strain *Xp* GEV2013 has chromosomal integration of copper resistance.

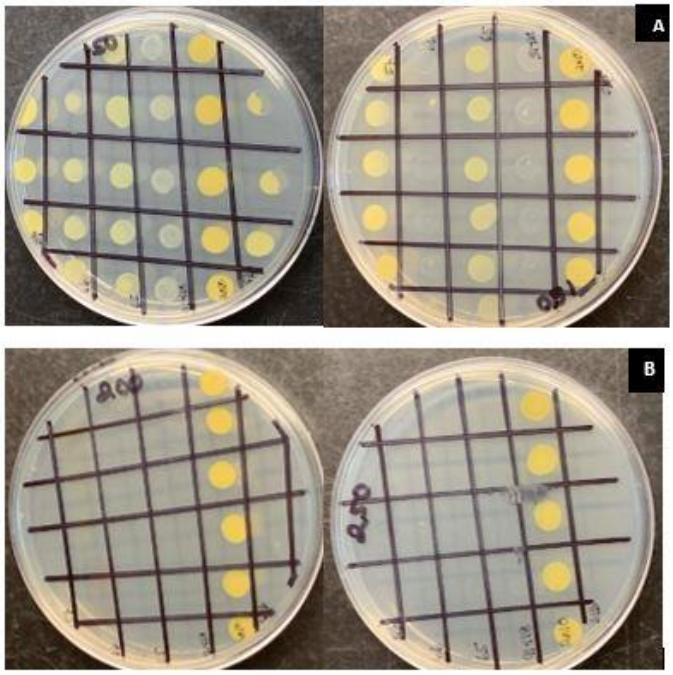

Figure 3-6: Growth of different *Xanthomonas* strains on copper amended nutrient agar medium 24 h after plating. A) *Xanthomonas* strains *Xp* AL57. *Xp* Al 66, *Xp* AL65, *Xp* 91-118, *Xp* 2010 and *Xp* ALS7B (from left to right) on nutrient agar amended with 50 ppm (left) and 150 ppm(right) of copper. B) *Xanthomonas* strains *Xp* AL57. *Xp* Al 66, *Xp* AL65, *Xp* 91-118, *Xp* 2010 and *Xp* ALS7B (from left to right) on nutrient agar amended with 200 ppm (left) and 250 ppm(right) of copper.

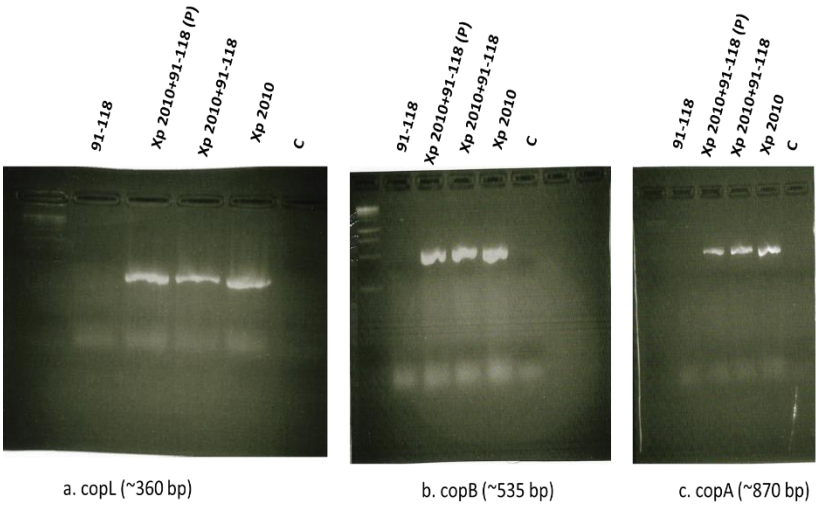

Figure 3-7: PCR amplification of copper resistance gene *copA, copB* and *copL* for colonies from *in planta* experiment showing the positive amplification

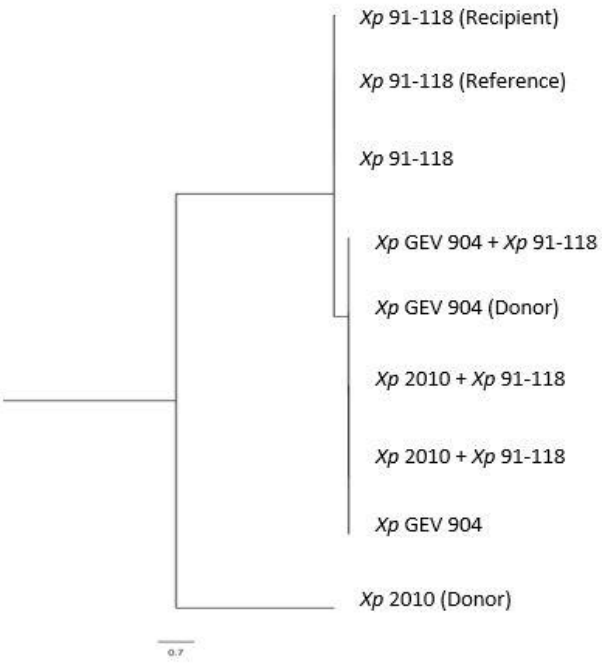

Figure 3-8: Midpoint-rooted phylogenetic tree based on single-nucleotide polymorphisms (SNPs) in the core genome of different donor, recipient and strains from *in planta* experiment. Strains were mapped against the reference genome of *Xp* 91-118 using on Parsnp program (Treangen et al. 2014).

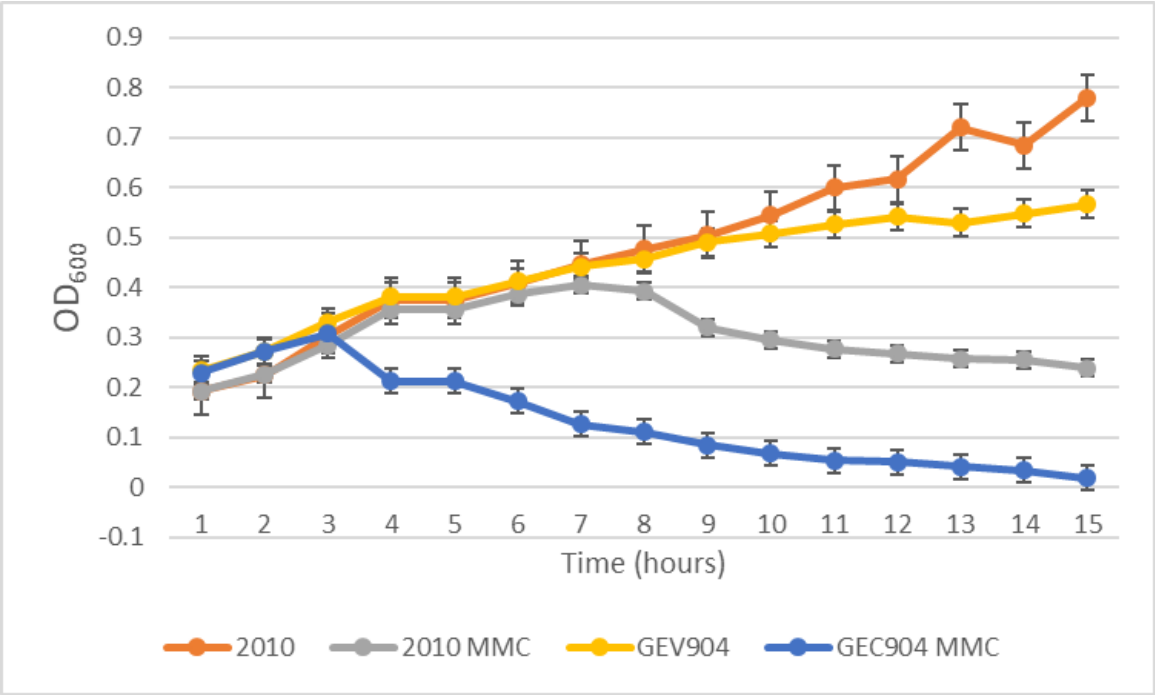

Figure 3-9: Growth curve of *Xp* 2010 and *Xp* GEV904 cultures showing a decline in optical density indicative of lysogeny with treated after addition of mitomycin C (0 h).

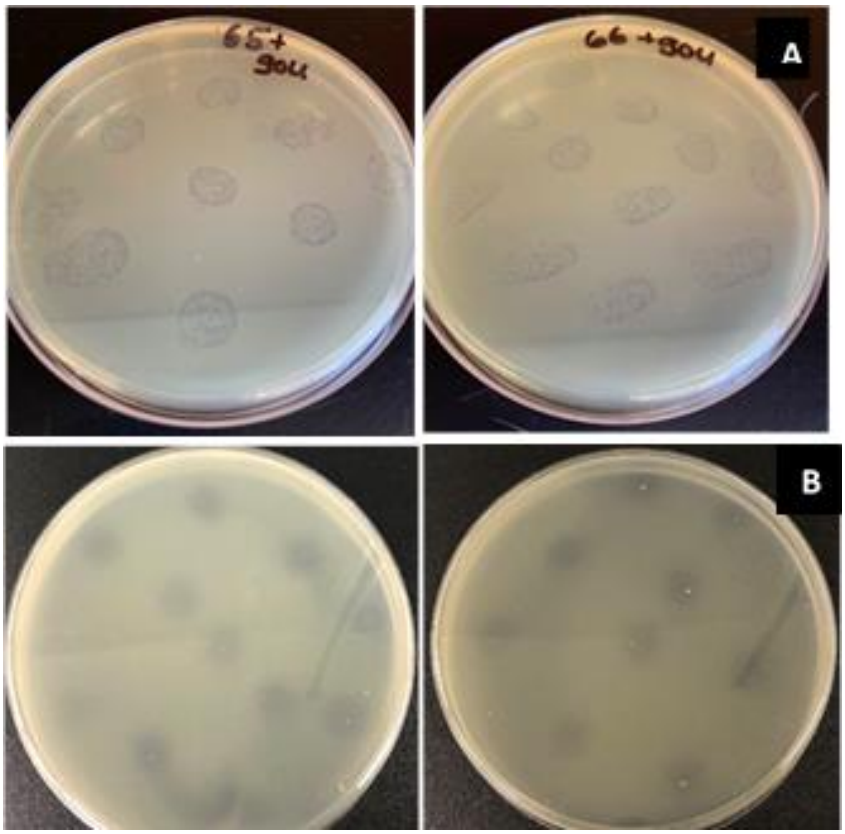

Figure 3-10: Plaque plate assay for test of antagonism by donor strains to different strains to be used as a recipient. A) recipient strains *Xp* AL65 and *Xp* AL66 showing a turbid plaque with donor strain *Xp* GEV 904. B) Recipient strain *Xp* 91-118 showing positive plaque test for donor *Xp* 2010.

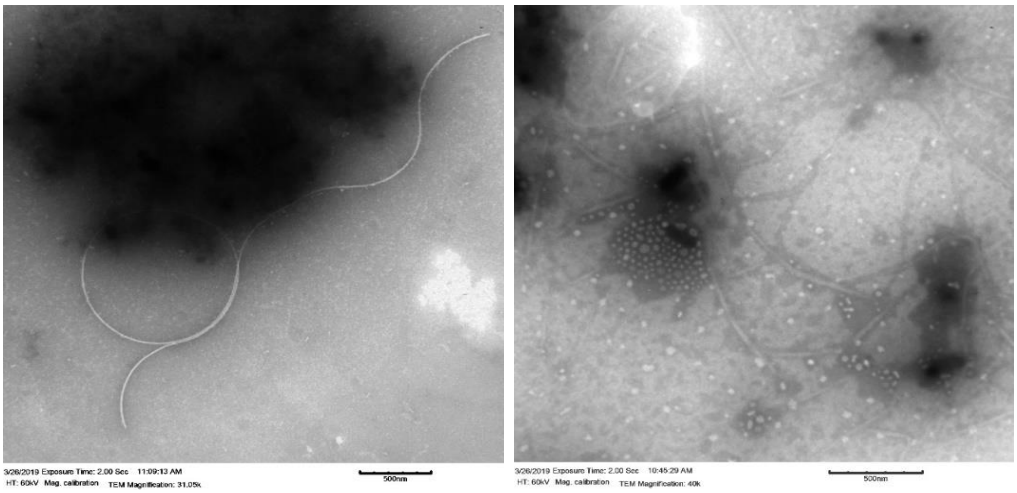

Figure 3-11: Transmission electron microscopy (TEM) of phage lysate from *Xp* 2010 (left) and *Xp* GEV 904 (right) showing elongated phage.

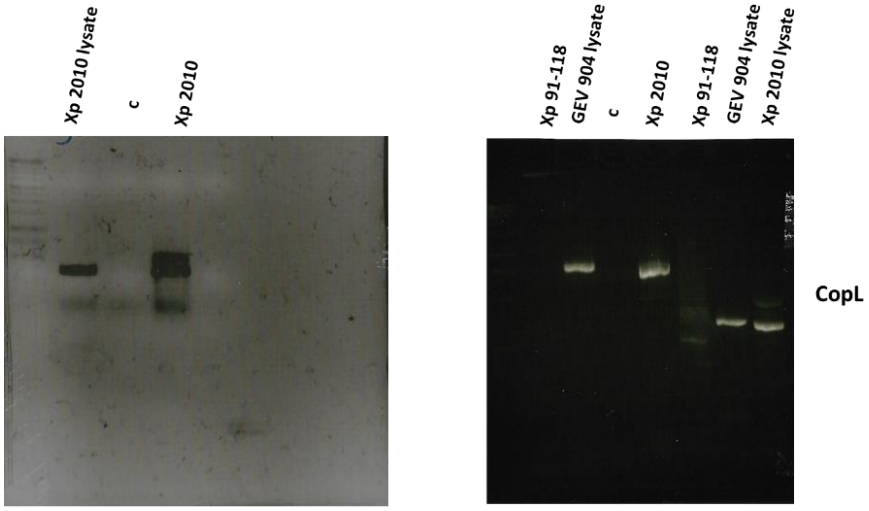

Figure 3-12: PCR amplification of copper resistance gene *copA* for *Xp* 2010 phage lysate (left) and *Xp* GEV904 (Right) and *copL* (Right) in phage DNA extracted from lysate from *Xp* 2010 and *Xp* GEV 904.

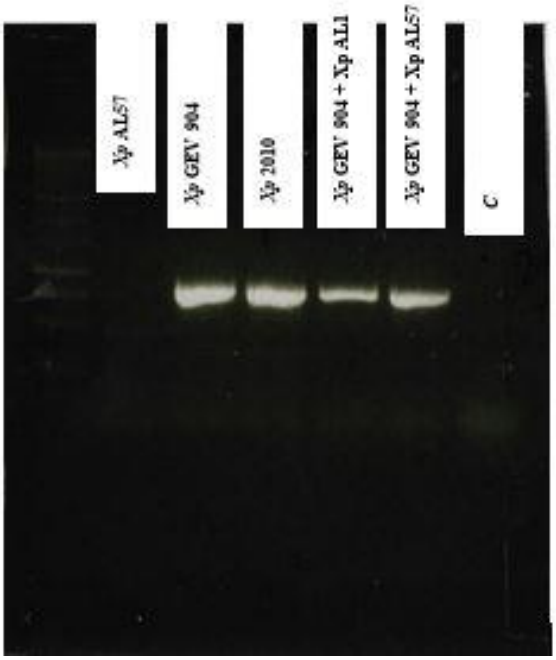

Figure 3-13: PCR amplification of copper resistance gene *copA* for colonies obtained from in-vitro experiment. Strain *Xp* 2010 is used as a positive control for copper resistance. C is used as a water control.

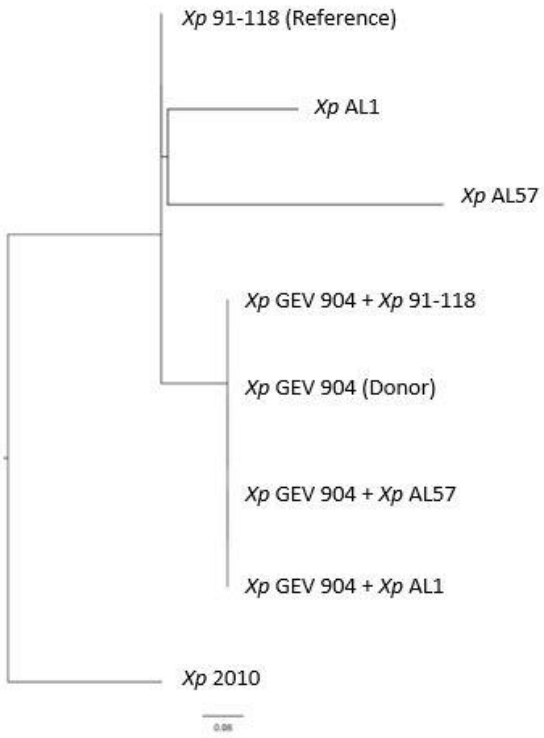

Figure 3-14: Midpoint-rooted phylogenetic tree based on single-nucleotide polymorphisms (SNPs) in the core genome of different donor, recipient and strains from *in-vitro* experiment. Strains were mapped against the reference genome of *Xp* 91-118 using on Parsnp program (Treangen et al. 2014).

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