POPULATION DIVERSITY AND *IN PLANTA* MOVEMENT OF *CLAVIBACTER MICHIGANENSIS* SUBSP. *MICHIGANENSIS*

A Dissertation

Presented to the Faculty of the Graduate School

of Cornell University

in Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

by

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August 2016

ABSTRACT

The Gram-positive bacterium *Clavibacter michiganensis* subsp. *michiganensis* is the causal agent of bacterial canker of tomato, and an economically devastating seed-borne pathogen that inflicts considerable damage throughout all major tomato producing regions. Within New York, annual outbreaks are a common occurrence since *C. michiganensis* subsp. *michiganensis* is easily spread by infected transplants, improper sanitation, and/or contaminated seed. In order to better characterize the pathogen and its interactions with tomato, my research focused on population diversity and the *in planta* movement of *C. michiganensis* subsp. *michiganensis*, with an emphasis on virulence and modes of infection. The first chapter explores the diversity of the *C. michiganensis* subsp. *michiganensis* New York population. Over an eleven year period, 51 field isolates were collected from independent outbreaks throughout New York and characterized with both repetitive element-PCR and multilocus sequence analysis. Our research identified a highly diverse New York *C. michiganensis* subsp. *michiganensis* population. Several of the isolates were reoccurring strains, but many were novel and appeared on farms with no previous history of bacterial canker of tomato, suggesting recent introduction events from infected seed or transplants. The second chapter investigates the routes of seed infection by using a constitutively eGFP-expressing virulent *C. michiganensis* subsp. *michiganensis* isolate (GCMM-22) to demonstrate that *C. michiganensis* subsp. *michiganensis* does not only access seeds systemically through the xylem, but also externally through tomato fruit lesions, which harbor high intra-and intercellular populations. *C. michiganensis* subsp. *michiganensis* was observed within the developing seed, endosperm, and funiculus, although at relatively low levels compared to the large number of cells observed in the xylem and pericarp cells. Chapter 3 characterizes the

putative plant-like expansin gene, *CmEXLX2*, in order to elucidate its role in disease development. Disruption of *CmEXLX2* in *C. michiganensis* subsp. *michiganensis* resulted in significant increases in necrosis, atypical lesion proliferation, and symptom development on tomato, compared to the wild-type and complemented strains. No differences in growth were observed *in vitro* between strains, but larger *in planta* mutant populations appeared to be directly associated with increased disease severity. We hypothesize that increased necrosis within the nutrient-poor xylem may result in increased nutrient availability, yielding larger *in planta* mutant populations and magnifying symptom progression. Finally, included in the appendix is a preliminary molecular study investigating the role of a putative β-N-acetylglucosaminidase (*nagA*) in *C. michiganensis* subsp. *michiganensis* infections. Proteomic studies demonstrated that β-N-acetylglucosaminidase was the third most upregulated protein under infection-mimicking conditions, but its role in infection remains unknown. Disruption of *nagA* resulted in the reduction of bacterial attachment *in vitro* when grown in tomato sap.

BIOGRAPHICAL SKETCH

In 2011, Matthew A. Tancos received a Bachelor of Science in Biology (concentrations in genetics and ecology) from Ball State University in Muncie, Indiana under the direction of Dr. Robert Hammersmith. During his undergraduate, Matthew gained research experience with a variety of institutions including: (i) a Cornell University nanobiotechnology internship researching pathogen diagnostics with Drs. Christine Smart and Harvey Hoch at the New York State Agricultural Experiment Station, (ii) a U.S. Environmental Protection Agency internship researching the microbiological treatment of drinking water for arsenic removal with Dr. Darren Lytle, and (iii) at Ball State University characterizing the ciliated protozoan *Oxytricha fallax* with Dr. Robert Hammersmith. Upon his 2011 graduation, Matthew began his Ph.D in Plant Pathology and Plant-Microbe Biology at Cornell University under the guidance of Dr. Christine Smart, for which he received a National Science Foundation Graduate Research Fellowship in 2013.

Dedicated to my loving and supportive wife Kiersten and my ever encouraging family

ACKNOWLEDGEMENTS

First and foremost I would like to thank my advisor, Dr. Christine Smart, for introducing me to the field of plant pathology, accepting me into her program, guiding and encouraging my scientific questions, and for providing the ideal environment for fostering scientific growth and curiosity. I would also like to thank the entire Smart Lab community – Holly Lange, Amara Dunn, Lisa Jones, Carly Summers, Zach Hansen, Rachel Kreis, Maryn Carlson, Chase Crowell, and Frank Peritore – for all of their patience, guidance, friendship and hilarity. Thank you to committee members Drs. Alan Collmer, Bill Fry, and Marc Fuchs for your time, guidance, honesty, and encouragement. Thank you to the undergraduate summer scholars who honored me with their scientific curiosity and friendships including: Shaun Stice, Chase Crowell, Alden Brewer, Gretchen Dykes, Anjali Merchant, and Nathan Martin. I am also thankful for the faculty in the Section of Plant Pathology & Plant Microbe Biology, especially everyone at the NYSAES including Dr. Harvey Hoch. Finally I would like to thank all of my funding sources including: Cornell University, American Phytopathological Society, United States-Israel Binational Agricultural Research and Development (BARD) grant (IS-4403-11C), and the NSF pre-doctoral fellowship (DGE-1144153).

Last but not least, I would like to thank my wife Kiersten Tancos for all of her encouragement, love, and support; and my mom and family for instilling in me the importance of hard work, persistence, respect, and the importance of always remaining curious and appreciating the beauty in life and nature.

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INTRODUCTION

Bacterial canker of tomato, caused by the seed-borne pathogen *Clavibacter michiganensis* subsp. *michiganensis*, is an economically devastating disease that inflicts considerable damage throughout all major tomato producing nations (de León et al. 2011). My thesis research focused on the movement of *C. michiganensis* subsp. *michiganensis* both geographically and microscopically, in order to characterize population diversity, modes of seed infection, and genes that modify virulence. Important epidemiological factors occur not only at the 'macro' scale, but also the 'micro' scale, and in order to acquire a complete understanding of a disease, both levels need to be addressed.

The *Clavibacter* genus is comprised entirely of phytopathogens with only the single species *michiganensis* being represented by five subspecies. *Clavibacter michiganensis* has a wide host range of both monocots and dicots, including: tomato (subsp. *michiganensis*), potato (subsp. *sepedonicus*), alfalfa (subsp. *insidiosus*), wheat (subsp. *tessellarius*), and maize (subsp. *nebraskensis*). The center of origin for *C. michiganensis* remains unknown, but presumably originated in the Americas since three of the five hosts are native to the Americas, and non-pathogenic *C. michiganensis*-like strains have been characterized from the endophytic bacterial populations of North American prairie plants (Zinniel et al. 2002). Recent phylogenetic studies further support this hypothesis because non-pathogenic *C. michiganensis* strains isolated from tomato seeds, collected only from California and Chile, are genetically unique compared to pathogenic strains of *C. michiganensis* subsp. *michiganensis* (Yasuhara-Bell and Alvarez 2015).

Clavibacter michiganensis subsp. *michiganensis* was initially isolated from Grand Rapids Michigan (USA) by Erwin F. Smith in 1909, with the disease rapidly spreading to nearby states

including New York (Smith 1910; Bryan 1930). Bacterial canker of tomato appeared to be confined to the North/Northeastern US; however, a severe outbreak occurred throughout New York in 1926, and by 1927 it was reported in the Southern, Western, and Midwestern US (Jones and Pederson 1928; Bryan 1930). During the same period, outbreaks of bacterial canker of tomato began to be reported internationally (Bryan 1930). Even though *C. michiganensis* subsp. *michiganensis* was first characterized in Michigan (the basis for its name), it is unlikely to have originated in the state. Instead, Michigan became the "founding" site of bacterial canker of tomato because this was where the renowned bacteriologist Erwin F. Smith performed his scholastic studies.

Due to its destructive nature, *C. michiganensis* subsp. *michiganensis* is designated as an A2 quarantine pest (present/detected in some parts of the region) for Europe, Asia, the Caribbean, and Africa (CABI and EPPO 1999; EFSA PLH Panel (EFSA Panel on Plant Health) 2014; de León et al. 2011). *C. michiganensis* subsp. *michiganensis* is known to enter the tomato epiphytically, such as through natural openings or wounds, or through infected seed. Once inside a plant, this vascular-inhabiting Gram-positive bacterium multiplies in the xylem, forming extensive biofilm-like structures, which lead to systemic infections (Chalupowicz et al. 2012) . Eventually, high titers result in the degradation of xylem and the subsequent spread into adjacent vessels and parenchyma cells; thereby, leading to the characteristic wilting, marginal necrosis of leaflets, and stem cankers (Wallis 1977). In addition to entering through the foliage, *C. michiganensis* subsp. *michiganensis* is able to colonize immature green fruit through glandular and nonglandular trichomes, which begin to shed as the fruit matures, resulting in characteristic 'bird's-eye' lesions (Getz et al. 1983; Bryan 1930).

Bacterial canker of tomato is difficult to control due to the lack of resistant cultivars and improper sanitation techniques (Werner et al. 2002; Sen et al. 2013). Furthermore, latent infections and symptomless seedlings are relatively common in both the greenhouse and field, which make *C. michiganensis* subsp. *michiganensis* outbreaks even more insidious (Gitaitis et al. 1991; Werner et al. 2002; Chang et al. 1991). Secondary spread predominantly occurs epiphytically by splashing water (entering through hydathodes) or mechanically with tools, trellising stakes, greenhouse benches or workers (Carlton et al. 1998; Chang et al. 1991; Werner et al. 2002).

Unfortunately, outbreaks of bacterial canker continue to occur throughout the approximately 3,000 acres of annually grown fresh market tomatoes (*Solanum lycopersicum*) in New York (USDA 2013). Therefore, my first chapter focuses on characterizing the *C. michiganensis* subsp. *michiganensis* population present within New York. Our hypothesis was that the New York *C. michiganensis* subsp. *michiganensis* population was highly diverse with both novel and persistent haplotypes being present. The objectives were to screen New York *C. michiganensis* subsp. *michiganensis* isolates, collected over eleven years, to determine (i) the number of unique haplotypes, (ii) how the haplotypes were spatially and temporally separated, and (iii) whether haplotypes were persisting on farms. We also sought to characterize virulence and plasmid diversity, and to evaluate the importance of putative virulence genes. With this basic understanding of the New York population, we could then compare our strains to *C. michiganensis* subsp. *michiganensis* strains characterized globally, and determine if external haplotypes were still a major problem to New York tomato production.

Contaminated tomato seed continues to remain one of the underlying reasons for global and regional *C. michiganensis* subsp. *michiganensis* outbreaks (de León et al. 2011). My second

chapter focuses on understanding the routes of seed infection. Phytopathogens actively access their hosts' seed through three dominant routes: systemically through the vasculature, externally by penetrating the ovary wall, or through floral parts (Agarwal and Sinclair 1997; Singh and Mathur 2004). As opposed to natural routes of infection, infected seed could also develop from improper seed treatment and handling procedures resulting from diseased plant tissue or contaminated machinery. Thereby spreading the phytopathogen during the extraction process and superficially contaminating seed batches.

Since *C. michiganensis* subsp. *michiganensis* is a systemic pathogen, it is hypothesized that it can access the seed through the hosts' vascular system. However, no detailed histopathological studies have clearly demonstrated this or other natural routes of seed infection (Bryan 1930; Singh and Mathur 2004). For example, bird's eye lesions are believed to be superficial blemishes that develop in the field when bacteria are splashed onto the developing fruit, with minimal impact on fruit colonization or seed infection. Our hypothesis was *C. michiganensis* subsp. *michiganensis* could contaminate seed through multiple routes of entry. Testing this hypothesis required a stable, virulent, and constitutively expressing eGFP strain, which was necessary to elucidate the pathogen's movement and seed internalization patterns over an extended period. Our objectives were to test the ability of *C. michiganensis* subsp. *michiganensis* to colonize developing tomato fruit and seed either systemically through the xylem and/or externally through lesions on the fruit pericarp.

Few studies have looked at the ability of other *C. michiganensis* subspecies to colonize true seed. *Clavibacter michiganensis* subsp. *insidiosus* (causes bacterial wilt of alfalfa) could be isolated from discolored and shriveled alfalfa seed, but was rarely observed in the seed even with large pedicel populations. When *C. michiganensis* subsp. *insidiosus* was observed, it was

predominantly localized to the aleurone layer and seed coat (Cormack and Moffatt 1956). *Clavibacter michiganensis* subsp. *nebraskensis* (causes Goss's wilt of corn) was shown to subsist in the chalazal region (within the vicinity of the embryo) of heavily discolored seeds with bacterial exudate (Schuster 1972; Biddle et al. 1990). However, when another study tried to observe seed-to-seedling transmission rates, no naturally infected seed led to seedling infection (Biddle et al. 1990). Lastly, *C. michiganensis* subsp. *tessellarius* (causal agent of bacterial mosaic of wheat) was observed with scanning electron microscopy in the seed coat and endosperm of infected wheat seed (McBeath and Adelman 1986). Overall, all *C. michiganensis* subspecies have been associated with seed infections. Therefore, elucidating the multifaceted infection process of *C. michiganensis* subsp. *michiganensis* could increase our understanding of Gram-positive phytopathogenic bacteria, and possibly provide insight into seed infection mechanisms that could aid in needed control measures.

Successful colonization of the xylem and subsequent spread into the developing fruit vasculature requires an ever-changing host-pathogen relationship and the ability of *C. michiganensis* subsp. *michiganensis* to suppress or evade plant defenses. Therefore, my third chapter investigates how *C. michiganensis* subsp. *michiganensis* is able to systemically spread, relatively unabated, within tomato. Colonization of the tomato xylem results in systemic infections with symptoms becoming evident when *in planta* populations ($\geq 10^8$ CFUs/g of tissue) impair water transpiration and macerate tissues leading to wilting and canker development, usually within a few weeks depending upon plant and environmental factors (Chang et al. 1992; Sharabani et al. 2013, 2014). *Clavibacter michiganensis* subsp. *michiganensis* utilizes numerous secreted cell-wall-degrading-enzymes (xylanase, polygalacturonase, pectate lyase, and endoglucanases), serine proteases, and tomatinase to successfully colonize tomatoes (Gartemann

et al. 2008; Savidor et al. 2012). Many of these putative pathogenicity and/or virulence genes are encoded by two plasmids (pCM1 and pCM2) and a 129 kb low GC content pathogenicity island (Gartemann et al. 2008). Unfortunately, understanding the role of virulence factors at the molecular level remains limited, but numerous *C. michiganensis* subsp. *michiganensis* polypeptides are known to be up-regulated under infection-mimicking conditions (Savidor et al. 2012). Currently, only four putative *C. michiganensis* subsp. *michiganensis* virulence genes have been investigated in detail, including: *pat-1* (serine protease), *celA* (β-1,4-endoglucanase), *tomA* (tomatinase), and *chpC* (serine protease), but the targets or precise molecular roles of Pat-1, CelA, and ChpC remain speculative (Dreier et al. 1997; Jahr et al. 2000; Kaup et al. 2005; Stork et al. 2008). To further increase our understanding of *C. michiganensis* subsp. *michiganensis*-tomato interactions, the two putative virulence genes β-N-acetylglucosaminidase (*nagA*) and a plant-like expansin (*CmEXLX2*) were selected to be disrupted in *C. michiganensis* subsp. *michiganensis* strains from New York.

Clavibacter michiganensis subsp. *michiganensis* and a small number of vascular-colonizing phytopathogenic bacteria possess putative expansin proteins that share high structural similarity to plant expansins and are hypothesized to have been horizontally acquired independently from plant hosts (Nikolaidis et al. 2014; Georgelis et al. 2014). Plant expansins are known to be localized to the cell wall and necessary for cell wall loosening through an uncharacterized pH-dependent mechanism; however, the function of bacterial expansins and their potential role in pathogenesis remains unknown (Cosgrove 2000; Kerff et al. 2008). The plant-like expansin *CmEXLX2* was disrupted in the New York field strain Cmm0317. We hypothesized that *CmEXLX2* was an important virulence factor in *C. michiganensis* subsp. *michiganensis* and necessary for successful xylem colonization. Our objectives were to (i)

explore the role of CmEXLX2 in disease progression and symptom development of tomato and (ii) determine the role CmEXLX2 has on vascular colonization and systemic movement. The putative role of *nagA* in pathogenesis was additionally investigated due to its presence in the pathogenicity island of *C. michiganensis* subsp. *michiganensis* and its increased expression under *in vitro* infection-mimicking conditions (Savidor et al. 2012). The function of the β-N-acetylglucosaminidase protein remains unknown, but has been associated with attachment in other bacterial systems (Bateman et al. 2005). Elucidating molecular functions necessary for xylem infections will not only increase our understanding of the *C. michiganensis* subsp. *michiganensis*-tomato pathosystem, but also of Gram-positive phytopathogenic bacteria.

Fundamental advances in the understanding of how *C. michiganensis* subsp. *michiganensis* colonizes tissue (fruit, seed, and vasculature) are needed to develop novel strategies to help reduce the pathogen's continued dissemination. My thesis objectives are divided into three chapters: (i) characterizing the New York *C. michiganensis* subsp. *michiganensis* population, (ii) understanding the natural routes of seed infection and (iii) investigating how *C. michiganensis* subsp. *michiganensis* is able to systemically colonize the tomato xylem.

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CHAPTER 1

CHARACTERIZING THE GENETIC DIVERSITY OF THE *CLAVIBACTER MICHIGANENSIS* SUBSP. *MICHIGANENSIS* POPULATION IN NEW YORK[1]

ABSTRACT

New York *Clavibacter michiganensis* subsp. *michiganensis* isolates, collected from disparate bacterial canker of tomato outbreaks over the past eleven years, were characterized with a multilocus sequence analysis (MLSA) scheme that differentiated the 51 isolates into 21 haplotypes with a discriminatory power of 0.944. The MLSA scheme consisted of five housekeeping genes (*kdpA*, *sdhA*, *dnaA*, *ligA*, *gyrB*) and three putative virulence genes (*celA*, *tomA*, *nagA*). Repetitive-PCR, with the BOX-A1R primer, confirmed the high diversity of *C. michiganensis* subsp. *michiganensis* isolates in New York by demonstrating all six PCR patterns (A, B, 13C, 65C, 81C, D) were present, with PCR patterns 'C' and 'A' being the most common. The MLSA scheme provided higher resolving power than the current repetitive-PCR approach. The plasmid profiles of New York isolates were diverse and differed from reference strain NCPPB382. PCR analysis indicated that the presence of putative virulence genes varied between isolates and highlighted the ephemeral nature of virulence genes in field populations of *C. michiganensis* subsp. *michiganensis*. Analysis of molecular variance between Serbian and New York *C. michiganensis* subsp. *michiganensis* isolates demonstrated that the two populations

[1] Reprinted from Tancos, M.A., Lange, H.W., and Smart, C.D. 2015. Characterizing the genetic diversity of the *Clavibacter michiganensis* subsp. *michiganensis* population in New York. *Phytopathology* 105:169-179.

were not significantly different, with 98% genetic variation within each population and only 2% genetic variation between populations.

INTRODUCTION

Clavibacter michiganensis subsp. *michiganensis*, the causal agent of bacterial canker of tomato (*Solanum lycopersicum*), is a quarantined phytopathogen in Europe, Asia, Africa, and the Caribbean (de León et al. 2011). This Gram-positive bacterium is present in many tomato-producing regions, and can rapidly spread globally through contaminated seed (Tancos et al. 2013; de León et al. 2011). *C. michiganensis* subsp. *michiganensis* spreads systemically throughout the host's vascular system via the xylem, and radiates outwards into the surrounding tissue when high titers are reached (Chalupowicz et al. 2012; Tancos et al. 2013). Once introduced, *C. michiganensis* subsp. *michiganensis* spreads epiphytically by splashing water or mechanically via workers and/or equipment (Carlton et al. 1998; Bryan 1930). Bacterial canker of tomato can be difficult to control due to the lack of resistant cultivars, ineffective chemical controls, and poor sanitation practices (Werner et al. 2002; Sen et al. 2013).

Based upon the genome sequence of reference strain NCPPB382, *C. michiganensis* subsp. *michiganensis* possesses an arsenal of secreted serine proteases and cell-wall-degrading enzymes, which are located within a chromosomal pathogenicity island (PAI) and two plasmids (pCM1 & pCM2) (Gartemann et al. 2008; Eichenlaub and Gartemann 2011; Balaji et al. 2008). The PAI is approximately 129 kb with a low GC content (64-66%), relative to the genome (72.7%), and is subdivided into two regions termed the *chp* and *tomA* subregions (Gartemann et al. 2008; Stork et al. 2008; Meletzus et al. 1993). The conjugative plasmids, pCM1 (27 kb) and pCM2 (70 kb) can be transferred throughout a population (Chalupowicz et al. 2010). The

current functions or targets of proteins produced by many of the putative pathogenicity genes remain undefined; however, they are hypothesized to function in colonization, nutrient acquisition, and/or host-defense suppression (Eichenlaub and Gartemann 2011; Chalupowicz et al. 2010; Stork et al. 2008).

The majority of *C. michiganensis* subsp. *michiganensis* studies have predominately focused on the pathogenic nature of the sequenced strain NCPPB382 (Meletzus et al. 1993; Gartemann et al. 2008; Chalupowicz et al. 2012); however, studies have recently begun to look at the diversity of field isolates, with differences such as plasmid number and presence/absence of putative virulence genes being readily observed with highly virulent field isolates (Quesada-Ocampo et al. 2012; Yasuhara-Bell et al. 2013; Milijašević-Marčić et al. 2012; Kleitman et al. 2008; Jacques et al. 2012; Nazari et al. 2007). Due to a deeper understanding of field diversity, diagnostic approaches have been tailored to reduce false-negatives by detecting diverse virulent and avirulent *C. michiganensis* subsp. *michiganensis* isolates collected from seed and tissue (Yasuhara-Bell et al. 2013).

DNA fingerprinting techniques like repetitive sequence-based polymerase chain reaction (rep-PCR) have been the predominant method used to characterize and investigate the epidemiology of *C. michiganensis* subsp. *michiganensis* populations internationally (de León et al. 2009; Kleitman et al. 2008; Kawaguchi et al. 2010; Nazari et al. 2007; Quesada-Ocampo et al. 2012; Louws et al. 1998). Within rep-PCR, the BOX-A1R primer is commonly able to differentiate *C. michiganensis* subsp. *michiganensis* isolates into four main PCR patterns (A, B, C, D), and the 'C' PCR pattern can be further segregated into three subgroups (13C, 65C, 81C) (Louws et al. 1998). However, the limited differentiation of isolates, incorrect identification of PCR patterns based on the subjectiveness of gels, and difficultly in inter-lab reproducibility

reduces the overall effectiveness of this technique. Conversely, sequencing approaches such as multilocus sequence analysis/typing (MLSA/MLST) and simple sequence repeats have provided a quick, efficient, and reproducible approach to microbial characterization, with whole genome sequencing quickly becoming the gold standard (Milijašević-Marčić et al. 2012; Köser et al. 2012; Zaluga et al. 2013).

Within New York, annual bacterial canker outbreaks occur throughout greenhouse and field environments; however, the diversity of *C. michiganensis* subsp. *michiganensis* isolates remains unexplored. The objectives of this study were to screen New York *C. michiganensis* subsp. *michiganensis* field isolates, which were collected over an eleven year period, with an MLSA scheme and (i) identify the diversity among isolates and (ii) compare the New York isolates to other *C. michiganensis* subsp. *michiganensis* populations.

METHODS AND MATERIALS

Bacterial isolates and growth conditions. The *C. michiganensis* subsp. *michiganensis* isolates utilized in the present study, except for 10-4R, were isolated from diseased tissue collected in New York during multiple bacterial canker outbreaks using D2ANX semi-selective media (Table 1.1, Figure 1.1) (Chun 1982). Isolate 10-4R was isolated from irrigation water in eastern New York (Jones 2014). All *C. michiganensis* subsp. *michiganensis* isolates were stored in 35% glycerol at -80°C.

Pathogenicity assays. Tomato seedlings (*Solanum lycopersicum*), cultivar Mountain Fresh Plus, were grown in a Cornell potting mix (composed of peat, perlite, and vermiculite in a 4:1:1 ratio) with a 14-hr light/ 10-hr dark photoperiod in the greenhouse. *C. michiganensis* subsp. *michiganensis* field isolates were grown for 72 hours on D2ANX agar plates and

Table 1.1: *Clavibacter michiganensis* subsp. *michiganensis* isolates used in this study

Isolate	NY county of origin	Co. symbol	Year collected	BOX-A1R pattern	Reference
0572	Ulster	ULT	2005	13C	This study
0582	Albany	ALB	2005	13C	This study
0651	Ulster	ULT	2006	13C	This study
0654	Ulster	ULT	2006	13C	This study
0676	Orange	ORG	2006	13C	This study
0690	Rensselaer	REN	2006	13C	(Tancos et al. 2013)
13109	Suffolk	SUF	2013	13C	This study
13115	Suffolk	SUF	2013	13C	This study
NCPPB382	United Kingdom	UK	1956	13C	NCPPB
CMM100	United Kingdom	UK	1956	13C	(Meletzus and Eichenlaub 1991)
04100	Schoharie	SCH	2004	65C	This study
04101	Schoharie	SCH	2004	65C	(Balaji and Smart 2012)
0748	Oneida	ONE	2007	65C	This study
0779	Oneida	ONE	2007	65C	This study
0780	Oneida	ONE	2007	65C	This study
0784	Rensselaer	REN	2007	65C	This study
0785	Albany	ALB	2007	65C	This study
09085	Niagara	NIG	2009	65C	This study
09086	Niagara	NIG	2009	65C	This study
10-4R	Rensselaer	REN	2010	65C	(Jones 2014)
11015	Albany	ALB	2011	65C	(Tancos et al. 2013)
12069	Clinton	CLT	2012	65C	This study
12084	Rensselaer	REN	2012	65C	This study
13054	Genesee	GEN	2013	65C	This study
13084	Wayne	WNY	2013	65C	This study
13091	Onondaga	OND	2013	65C	This study
0310	Washington	WAS	2003	81C	This study
0426	Washington	WAS	2004	81C	This study
0580	Washington	WAS	2005	81C	This study
0687	Washington	WAS	2006	81C	This study
0691	Washington	WAS	2006	81C	This study
0692	Washington	WAS	2006	81C	This study
0763	Orange	ORG	2007	81C	This study
0767	Oneida	ONE	2007	81C	(Tancos et al. 2013)
0775	Oneida	ONE	2007	81C	This study
08223	Ulster	ULT	2008	81C	This study
08224	Ulster	ULT	2008	81C	This study

09081	Niagara	NIG	2009	81C	This study
13129	Suffolk	SUF	2013	81C	This study
0312	Erie	ERI	2003	A	This study
0314	Erie	ERI	2003	A	This study
0315	Erie	ERI	2003	A	This study
0317	Ontario	ONT	2003	A	This study
0459	Erie	ERI	2004	A	This study
04108	Albany	ALB	2004	A	This study
06124	Albany	ALB	2006	A	This study
12083	Columbia	COL	2012	A	This study
13048	Yates	YAT	2013	A	This study
13117	Ontario	ONT	2013	A	This study
0581	Albany	ALB	2005	B	This study
04106	Schoharie	SCH	2004	D	This study
13090	Cayuga	CAY	2013	D	This study
13134	Washington	WAS	2013	D	This study

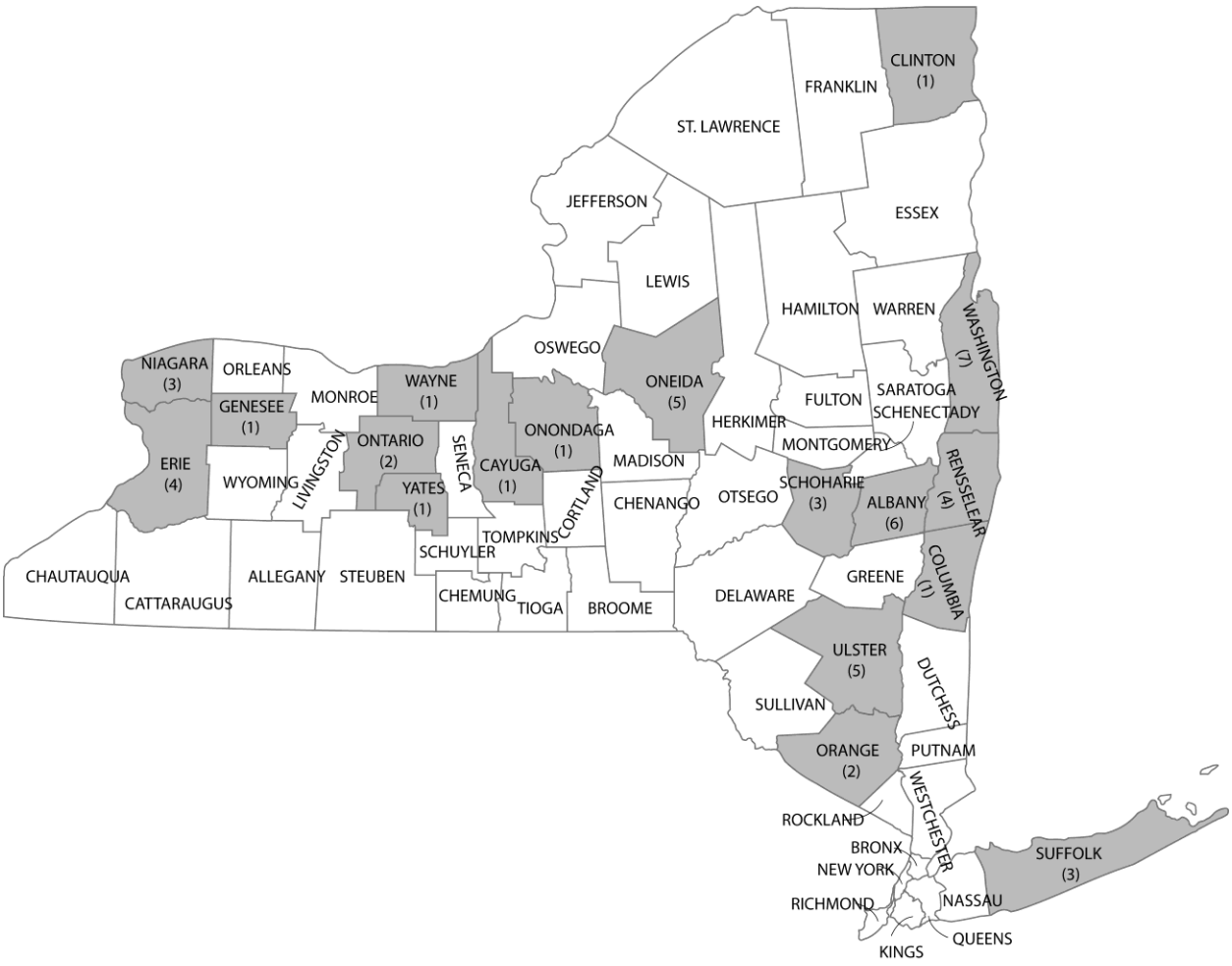

Figure 1.1: Map of New York with gray-colored counties representing locations of where isolates of *Clavibacter michiganensis* subsp. *michiganensis* were isolated. The number of isolates collected from each county is provided in parentheses (n=51).

inoculated into approximately three week old tomato seedlings (n=3/isolate) by pricking the stem between the cotyledons with a sterile needle dipped in an individual *C. michiganensis* subsp. *michiganensis* colony or sterile water as a negative control (Savidor et al. 2012). After the first week, tomato plants were screened daily for characteristic wilting and chlorosis associated with bacterial canker as previously described (1). Observations continued until all plants died or until 28 days post inoculation (dpi). Three tomato plants comprised a replicate, and the entire experiment was repeated for a total of 6 plants per isolate.

Plasmid profiles. To assay New York isolates for the presence of plasmids (n=36, plus NCPPB382 and CMM100), an individual *C. michiganensis* subsp. *michiganensis* CFU was grown at 27°C with shaking at 140 rpm for 36 hours in 30 ml of liquid LB, before being transferred to 500 ml of liquid LB for an additional 24 hours. Plasmid DNA was isolated with a Qiagen Large-construct kit (Qiagen, Enlo, Netherlands) according to the manufacturer's instructions, except 5 mg/ml of lysozyme was added to the resuspension buffer (reagent P1). All DNA was quantified with a Nanodrop ND-1000 (Wilmington, DE). Plasmid diversity was characterized using CHEF-DR II Pulsed Field Electrophoresis System (Bio-Rad Laboratories, Inc., Hercules, CA) using 6V/cm, 1% molecular biology grade agarose, 0.5x TBE, 0.5-10 sec linear ramp factor for a run time of 16 hours at 14°C. Plasmid DNA was used at 200 ng per well and visualized with ethidium bromide.

Genomic fingerprinting using BOX-A1R repetitive-PCR. Genomic DNA was extracted with the MasterPure Gram-Positive DNA purification kit (Epicentre, Madison, WI) according to the manufacturer's instructions. All DNA was quantified with a Nanodrop ND-1000 (Wilmington, DE). A fingerprint was generated for each isolate collected using the BOX-A1R primer following a previously described protocol (Louws et al. 1994, 1998) with a PTC-100 Peltier

thermal cycler (MJ Research, Waltham, MA). Reference *C. michiganensis* subsp. *michiganensis* strains for the six BOX-A1R patterns (A, B, 13C, 65C, 81C, and D) were included with the New York isolates. Extracted total DNA was used at 20 ng per reaction. PCR was performed in 25 µl reactions with the following parameters: initial preheat for 2 min at 95°C, 35 cycles at 94°C for 1 min, 53 ºC for 1 min, 65°C for 8 min, a final extension at 65°C for 15 min, and held at 10°C (Louws et al. 1994). The PCR products (8 µl) were run in a 1.5% agarose gel using 0.5X TAE buffer at 67 V for 18 hours at 4ºC.

MLSA of *C. michiganensis* subsp. *michiganensis*. Primers for the putative virulence genes – *celA, nagA, chpE, pat-1, phpA* – were designed using Primer3 v. 4.0.0 (Untergrasser et al. 2012; Koressaar and Remm 2007) and the sequenced *C. michiganensis* subsp. *michiganensis* NCPPB382 genome (Table 1.2). Primers targeting the housekeeping genes – *kdpA, sdhA, dnaA, ligA, gyrB* – and one additional putative virulence gene (*tomA*) had been previously described (Milijašević-Marčić et al. 2012; Zaluga et al. 2013; Richert et al. 2005). In order to enhance the discriminatory power of the initial MLST scheme published by Milijašević-Marčić and et al. (2012), additional genes were sequenced including putative virulence genes (Zaluga et al. 2013). Multi-virulence-locus sequencing typing including virulence genes has been commonly employed to subtype human bacterial pathogens due to the hypervariable nature of virulence genes, thereby increasing the resolving power of sequence analyses (Shariat et al. 2013; Zhang et al. 2004; Chen et al. 2007; Reid et al. 2001).

The eleven housekeeping and putative virulence genes were analyzed for each isolate using total DNA (as described above) and gene-specific PCR (Table 1.2). Amplification was performed in a C1000 Touch Thermal Cycler (Bio-Rad Laboratories, Inc., Hercules, CA) using EmeraldAmp GT PCR Master Mix (Takara Bio Inc., Otsu, Shiga, Japan). Extracted total DNA

was used at approximately 30 ng per reaction with 10 µM each of forward and reverse primers (Table 1.2). PCR was performed in 25 µl reactions with the following parameters: initial preheat for 3 min at 95°C, 35 cycles at 95°C for 30 sec, a primer pair specific temperature for 30 sec (Table 1.2), 72°C for 1 min, a final extension at 72°C for 5 min, and held at 12°C. PCR products were electrophoresed on 1% gels at 90V for 45 minutes, followed by staining with ethidium bromide. All negative PCR reactions were repeated at least twice.

PCR products were purified with a DNA Clean & Concentrator – 25 kit (Zymo Research Inc., Irvine, CA) according to the manufacturer's instructions. The cleaned PCR amplicons were sequenced in both directions on a 3730XL (Sanger) DNA Analyzer (Applied Biosystems) at the Cornell University Life Sciences Core Laboratories Center with the aforementioned primer sets. Our final MLSA scheme consisted of eight genes comprising five housekeeping genes – *kdpA*, *sdhA*, *dnaA*, *ligA*, *gyrB* – and three putative virulence genes – *celA*, *tomA*, *nagA* – (Table 1.2).

Sequence analysis. Raw sequences for each of the eight genes in the MLSA scheme for all 52 isolates were viewed with FinchTV (Geospiza, Inc., Seattle, WA) and manually edited according to peak quality. The sequences were then aligned with Muscle in MEGA 5.2 (Tamura et al. 2011) on an individual gene basis or concatenated. Gene(s) that were missing in a given isolate were treated as deletions in further analyses (Wicker et al. 2012). Consensus sequences were used to generate maximum-likelihood (ML) trees based on the Tamura-Nei model (Tamura and Nei 1993) of MEGA 5.2 using all sites with bootstrapping repetitions of 1000. Sequences from reference strain *C. michiganensis* subsp. *sepedonicus* NCPPB2137 were used as an outgroup (Bentley et al. 2008). Haplotypes were considered unique if the sequence differed by a single nucleotide.

Table 1.2: Primers used for multilocus sequence analysis

Gene	Sequence (5'-3')	Gene product	Locus Tag	Tm (C)	Amplicon size (bp)	Aligned fragment (bp)	Reference
KdpA	F:GTGCAGAACTTCGTCTCGG R:GAGCATCATGTTGATCATCG	K+-dependent ATPase	CMM_2751	60	693	670	(Milijašević-Marčić et al. 2012)
SdhA	F:CCTGGATGTTCGTGTACC R:GAGGACATGGAGTTCTTCC	Succinate dehydrogenase	CMM_0970	58	778	749	(Milijašević-Marčić et al. 2012)
DnaA	F:TACGGCTTCGACACCTTCG R:CGGTGATCTTCTTGTTGGCG	Replication initiation factor	CMM_0001	60	933	833	(Zaluga et al. 2013; Schneider et al. 2011)
LigA	F:GTTCGACGAGCTGAATGC R:CTCGACCTTCTCCATGAC	DNA ligase	CMM_1404	56	544	524	(Milijašević-Marčić et al. 2012)
GyrB	F:ACCGTCGAGTTCGACTACGA R:CCTCGGTGTTGCCSARCTT	DNA gyrase, subunit B	CMM_0006	60	525	501	(Zaluga et al. 2013; Richert et al. 2005)
CelA	F:GTAGGGCACGCATTTCAGAG R:CAATGTCCTTCTTCGCCAGG	Cellulase	pCM1_0020	58	1240	1126	This study
TomA	F:CGAACTCGACCAGGTTCTCG R:GGTCTCACGATCGGATCC	Tomatinase	CMM_0090	60	529	509	(Kleitman et al. 2008)
NagA	F:AGAACCTGACAACGGTAGCT R:GCTTCGGTTGTTTACGGGAA	β-N-acetylglucosaminidase	CMM_0049	60	796	769	This study

ChpE	F:CCTGACGCTTCTACTTCCGA R:ACGTTTGTGCTGGTCCATTC	Serine protease	CMM_0039	58	695	674	This study
Pat-1	F:TGTAGACCGTATAGCCCGTG R:CCTGAGACCTATTACCGCCC	Serine protease	pCM2_0054	55	850	791	This study
PhpA	F:TCTCGCGAATCAGCCCATAT R:CTCCTCTAGTCTTCACGCCC	Serine protease	pCM2_0053	58	709	688	This study

Discriminatory power was calculated using a web-based discriminatory power calculator (http://insilico.ehu.es/mini_tools/discriminatory_power/index.php). The population genetic structure of the Serbian and New York *C. michiganensis* subsp. *michiganensis* isolates were tested using analysis of molecular variance (AMOVA; $P < 0.05$) via GenAlEx 6.501 with 999 permutations (Peakall and Smouse 2012, 2006). The concatenated sequences – *ligA*, *kdpA*, *sdhA*, *tomA* – from seven Serbian *C. michiganensis* subsp. *michiganensis* isolates, representing each of the seven MLST groups, were compared to the same concatenated sequences from each of the sixteen housekeeping gene haplotypes observed with the New York *C. michiganensis* subsp. *michiganensis* isolates (Milijašević-Marčić et al. 2012). Only these four genes could be compared since they were used in both studies.

RESULTS

Pathogenicity of New York *C. michiganensis* subsp. *michiganensis* isolates. A total of 51 *C. michiganensis* subsp. *michiganensis* isolates were collected over eleven years (Table 1.1). Disease incidence in greenhouse inoculated plants was consistent, except for six isolates that were only capable of producing disease symptoms in ≤ four of the six tomato seedlings tested (Table 1.3). New York isolate 09085 resulted in weak disease symptoms in only one of six seedlings, while other isolates started showing symptoms as early as seven dpi. No wilting was observed on any of the six 09085-inoculated tomato seedlings, but some chlorosis was observed on one plant during the observation period (28 dpi). No wilting or chlorosis was observed with any of the plants inoculated with water (negative controls).

Characterization of plasmid profiles. Plasmid diversity was analyzed for a subset of the New York *C. michiganensis* subsp. *michiganensis* isolates (n=36), representing at least one

Table 1.3. Genetic diversity observed among New York *Clavibacter michiganensis* subsp. *michiganensis* isolates[a]

BOX-A1R Pattern	Isolate	Plasmid			Chromosome					PAI[b]			Plasmid Profile[c]	Disease Incidence[d]	Hap[e]
		pat-1	*celA*	*phpA*	*dnaA*	*sdhA*	*gyrB*	*ligA*	*kdpA*	*chpE*	*tomA*	*nagA*			
13C	NCPPB382	+	+	+	+	+	+	+	+	+	+	+	P1	5/6	H1
	0572	+	+	+	+	+	+	+	+	+	+	+	P11	6/6	H13
	0582	+	+	+	+	+	+	+	+	+	+	+	*	6/6	H14
	0676	+	+	+	+	+	+	+	+	+	+	+	P15	6/6	H15
	0651	+	+	+	+	+	+	+	+	+	+	+	P16	6/6	H15
	0654	+	+	+	+	+	+	+	+	+	+	+	P16	6/6	H15
	13115	+	+	+	+	+	+	+	+	+	+	+	*	6/6	H16
	13109	+	+	+	+	+	+	+	+	+	+	+	*	6/6	H16
	0690	+	+	+	+	+	+	+	+	+	+	+	P1	5/6	H21
	CMM100	-	-	-	+	+	+	+	+	+	+	+	P8	2/6	**
65C	09086	+	+	-	+	+	+	+	+	+	+	+	P2	6/6	H2
	0785	+	+	+	+	+	+	+	+	+	+	+	P1	6/6	H5
	11015	+	+	+	+	+	+	+	+	+	+	+	P1	6/6	H5
	12069	+	+	+	+	+	+	+	+	+	+	+	P6	6/6	H6
	13091	-	+	-	+	+	+	+	+	+	+	+	*	6/6	H10
	13054	+	+	+	+	+	+	+	+	+	+	+	*	6/6	H10
	0780	+	+	+	+	+	+	+	+	+	+	+	P12	6/6	H10
	0779	+	+	+	+	+	+	+	+	+	+	+	P9	6/6	H10
	0748	+	+	+	+	+	+	+	+	+	+	+	*	6/6	H16
	04100	+	+	+	+	+	+	+	+	+	+	+	*	6/6	H16
	04101	+	+	+	+	+	+	+	+	+	+	+	P15	6/6	H16

	13084	-	+	-		+	+	+	+	+		+	+	+	*	6/6	H17
	10-4R	-	-	-		+	+	+	+	+		+	+	+	P8	4/6	H19
	12084	-	-	+		+	+	+	+	+		+	+	+	P8	4/6	H19
	09085	+	+	-		+	+	+	+	+		-	+	-	P2	1/6	H20
	0784	+	+	+		+	+	+	+	+		+	+	+	P1	6/6	H22
	09081	+	+	+		+	+	+	+	+		+	+	+	P2	6/6	H2
	0310	+	+	+		+	+	+	+	+		+	+	+	*	6/6	H4
	0426	+	+	+		+	+	+	+	+		+	+	+	*	6/6	H4
	0767	+	+	+		+	+	+	+	+		+	+	+	P13	6/6	H4
	0580	+	+	+		+	+	+	+	+		+	+	+	P5	6/6	H4
	0687	+	+	+		+	+	+	+	+		+	+	+	P5	6/6	H4
81C	0692	+	+	+		+	+	+	+	+		+	+	+	P5	6/6	H4
	0691	+	+	+		+	+	+	+	+		+	+	+	P5	6/6	H4
	13129	+	+	+		+	+	+	+	+		+	+	+	*	6/6	H9
	0763	+	+	+		+	+	+	+	+		+	+	+	P4	4/6	H9
	0775	+	+	+		+	+	+	+	+		+	+	+	P4	6/6	H9
	08223	+	+	+		+	+	+	+	+		+	+	+	P3	6/6	H11
	08224	+	+	+		+	+	+	+	+		+	+	+	P3	6/6	H11
	0312	+	+	-		+	+	+	+	+		+	+	+	*	6/6	H2
	0315	+	+	-		+	+	+	+	+		+	+	+	*	6/6	H2
	0314	+	+	-		+	+	+	+	+		+	+	+	P2	6/6	H2
	04108	+	+	-		+	+	+	+	+		+	+	+	*	6/6	H7
A	0459	+	+	-		+	+	+	+	+		+	+	+	*	6/6	H7
	06124	+	+	-		+	+	+	+	+		+	+	+	P2	4/6	H7
	12083	+	+	-		+	+	+	+	+		+	+	+	P4	6/6	H7
	13048	+	+	+		+	+	+	+	+		+	+	+	P5	4/6	H8
	13117	+	+	+		+	+	+	+	+		+	+	+	P2	6/6	H12

	0317	+	-	-		+	+	+	+	+		+	+	+	P14	6/6	H18
B	0581	+	+	+		+	+	+	+	+		+	+	+	P5	6/6	H4
D	04106	+	+	+		+	+	+	+	+		+	+	+	P7	6/6	H3
	13134	+	+	+		+	+	+	+	+		+	+	+	P10	6/6	H6
	13090	+	+	+		+	+	+	+	+		+	+	+	P6	6/6	H6

[a] Symbols: + (shaded) = gene present, - (blank) = gene absent, * = plasmid isolation was not performed, and ** = CMM100 was not included in sequencing analyses since it is identical to *Clavibacter michiganensis* subsp. *michiganensis* strain NCPPB382, minus the two plasmids. Gene location based upon the reference *Clavibacter michiganensis* subsp. *michiganensis* strain NCPPB382.

[b] PAI = pathogenicity island

[c] Unique plasmid profile patterns for *Clavibacter michiganensis* subsp. *michiganensis* isolates determined with pulsed-field gel electrophoresis.

[d] Disease incidence expressed as the number of wilting plants/number of inoculated plants 28 days post inoculation.

[e] Hap = haplotype: based on the concatenated housekeeping and putative virulence genes.

from each BOX-A1R pattern (Table 1.3). NCPPB382 and CMM100 were used as positive and negative controls, respectively. A total of 16 unique plasmid patterns – P1 (n=5), P2 (n=6), P3 (n=2), P4 (n=3), P5 (n=6), P6 (n=2), P7 (n=1), P8 (n=3), P9 (n=1), P10 (n=1), P11 (n=1), P12 (n=1), P13 (n=1), P14 (n=1), P15 (n=2), P16 (n=2) – were observed in the New York population of *C. michiganensis* subsp. *michiganensis* (Table 1.3, Figure 1.2). The majority of New York *C. michiganensis* subsp. *michiganensis* isolate plasmid profiles were different from reference strain NCPPB382 (plasmid pattern P1).

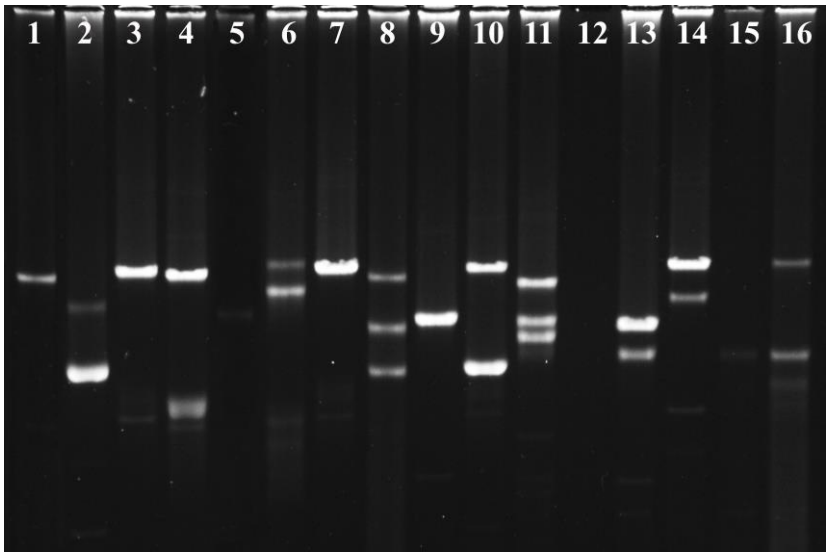

Figure 1.2: Pulsed-field gel electrophoresis plasmid profiles representing the most diverse plasmid patterns observed in the New York *Clavibacter michiganensis* subsp. *michiganensis* population. Lanes (1-16) : 1 = P14 (0317), 2 = P16 (0651), 3 = P2 (06124), 4 = P1 (NCPPB382), 5 = P15 (0676), 6 = P3 (08224), 7 = P4 (12083), 8 = P10 (13134), 9 = P6 (12069), 10 = P5 (13048), 11 = P7 (04106), 12 = P8 (CMM100), 13 = P9 (0779), 14 = P11 (0572), 15 = P12 (0780), 16 = P13 (0767).

BOX-A1R fingerprints. All six BOX-A1R patterns – A (n=10), B (n=1), 13C (n=8), 65C (n=16), 81C (n=13), D (n=3) – were observed in the New York population of *C. michiganensis* subsp. *michiganensis* within the past eleven years (Tables 1.1 & 1.3). The 'C' patterns were the most prominent groups observed, with at least one 'C' pattern isolated every year. However,

both the ‘B’ and ‘D’ patterns were rare with only one isolate collected in 2005, and three collected from 2004 to 2013, respectively (Table 1.1). Differentiation based on the six BOX-A1R patterns resulted in a discriminatory power of 0.785.

MLSA analyses for *C. michiganensis* subsp. *michiganensis*. The initial MLSA scheme was to consist of five housekeeping genes – *kdpA*, *sdhA*, *dnaA*, *ligA*, *gyrB* – and six putative virulence genes – *pat-1*, *celA*, *phpA*, *chpE*, *tomA*, *nagA* – (Table 1.4); however, no polymorphic sites were observed in the *pat-1*, *phpA*, or *chpE* genes when screened with diverse *C. michiganensis* subsp. *michiganensis* isolates (based on BOX-A1R analysis). The three genes with no polymorphic sites were not included in further phylogenetic analyses, but continued to be screened with PCR in order to determine presence or absence in each *C. michiganensis* subsp. *michiganensis* isolate (Table 1.3). The final MLSA scheme consisted of eight genes comprising five housekeeping genes – *kdpA*, *sdhA*, *dnaA*, *ligA*, *gyrB* – and three putative virulence genes – *celA*, *tomA*, *nagA* – (Table 1.2).

The MLSA scheme could resolve the 51 New York *C. michiganensis* subsp. *michiganensis* isolates into 21 haplotypes with a discriminatory power of 0.944, when the five housekeeping genes and three putative virulence genes were concatenated (Table 1.3). The most numerous haplotype (H) was H4 (n=8); followed by H2 and H16 (both n=5); H7 and H10 (both n=4); H6, H9, and H15 (all n=3); H5, H11, H19 (all n=2), and 10 unique haplotypes that were represented only once. Our MLSA scheme provided higher resolution than the BOX-A1R patterns because type ‘A’ could be further subdivided into 5 haplotypes, ‘13C’ into 5 haplotypes, ‘65C’ into 9 haplotypes, ‘81C’ into 4 haplotypes, ‘D’ into 2 haplotypes (Table 1.3). The isolate with the ‘B’ BOX-A1R pattern (0581) was a member of the H4 haplotype which included eight

Table 1.4. Sequence variation in *Clavibacter michiganensis* subsp. *michiganensis* isolates

Gene	Sequenced fragment (bp)	No. of isolates sequenced[a]	No. of haplotypes	Indel[b]	No. (%) of polymorphic sites	No. of polymorphic sites in noncoding sequences	Discriminatory Power
Housekeeping							
KdpA	670	52	12	0	25 (3.73%)	0	0.868
SdhA	749	52	13	0	32 (4.27%)	0	0.878
DnaA	833	52	4	0	8 (0.960%)	0	0.597
LigA	524	52	5	0	4 (0.763%)	0	0.283
GyrB	501	52	4	0	3 (0.599%)	0	0.489
Conc. KdpA-SdhA	1419	52	17	-	57 (4.02%)	-	0.934
Conc. KdpA-GyrB	3277	52	17	-	72 (2.20%)	-	0.934
Virulence							
CelA	1126	52	8	1	15 (1.33%)	3	0.806
TomA	509	52	5	0	4 (0.786%)	0	0.630
NagA	769	52	3	0	2 (0.260%)	0	0.185
ChpE	674	13	1	0	0	0	0
Pat-1	791	15	1	0	0	0	0
PhpA	688	4	1	0	0	0	0
Conc. CelA-NagA	2404	52	18	-	21 (0.874%)	-	0.905
Conc. KdpA-GyrB + CelA-NagA	5681	52	22	-	93 (1.64%)	-	0.946

[a] CMM100 was not included in sequencing analyses since it is identical to *Clavibacter michiganensis* subsp. *michiganensis* strain NCPPB382, minus the two plasmids.

[b] The number of unique indels detected within sequenced fragment. Each indel was treated as a single polymorphic site.

isolates. BOX-A1R patterns tended to cluster together within the ML phylogenetic trees; however, there was not an absolute association between BOX-A1R patterns and haplotypes (Figures 1.3 and 1.4). Different clustering patterns within the phylogenetic trees were observed when only the housekeeping genes were concatenated (Figure 1.3) versus the concatenating of both housekeeping genes and putative virulence genes (Figure 1.4).

All eight loci used in our MLSA scheme were polymorphic with *sdhA* having the most polymorphic sites at 32, and *nagA* having the least with only 2 polymorphic sites (Table 1.4). Housekeeping genes *kdpA* and *sdhA* showed the greatest amount of variation with 3.73% and 4.27% of sites being polymorphic in each gene, respectively (Table 1.4). The three remaining housekeeping genes – *dnaA*, *ligA*, *gyrB* – all had less than 1.0% of sequence variation. Concatenation of *kdpA* and *sdhA* alone produced the same number of haplotypes as concatenating all of the housekeeping genes in isolates of *C. michiganensis* subsp. *michiganensis* (Table 1.4). Putative virulence genes showed minimal sequence variation in comparison to the housekeeping genes of *C. michiganensis* subsp. *michiganensis* except for *celA* and *tomA*, which had 1.33% and 0.786% of sites being polymorphic in each gene, respectively (Table 1.4).

Polymorphic sites were predominately located within coding sequences, but three polymorphic sites (including an indel) were identified in the non-coding sequences of *celA* (Table 1.4). An identical 20 base pair (bp) insertion was located 17 bp upstream of the *celA* start codon in the six New York *C. michiganensis* subsp. *michiganensis* isolates: 04106, 0779, 0780, 0784, 13054, and 13091. All of the isolates except 0779 and 0780 originated in different New York counties located in disparate regions of New York including: western NY, central NY, the capital district, and eastern NY from 2004 to 2013 (Figure 1.1).

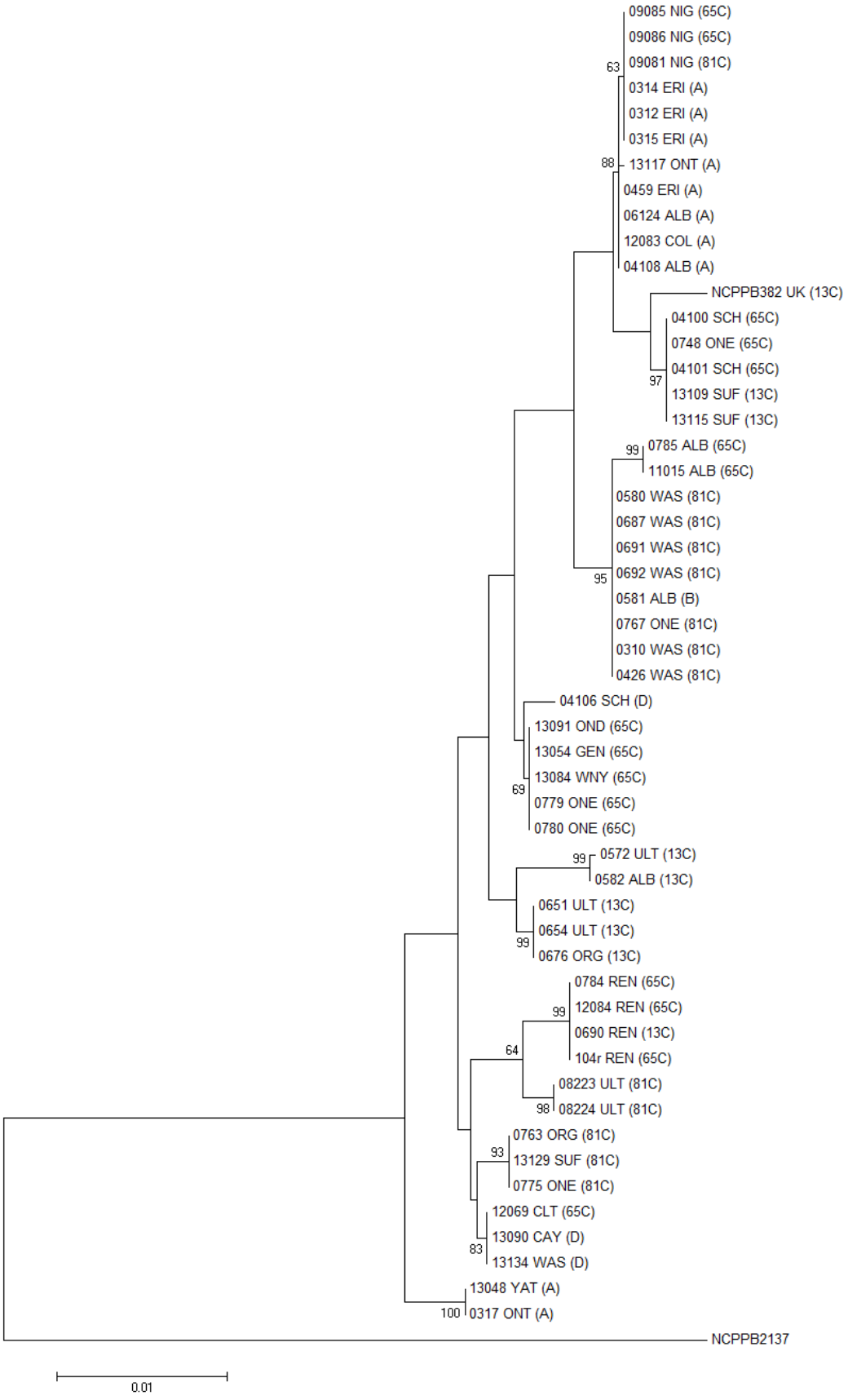
09085 NIG (65C)
09086 NIG (65C)
63
09081 NIG (81C)
0314 ERI (A)
0312 ERI (A)
0315 ERI (A)
88
13117 ONT (A)
0459 ERI (A)
06124 ALB (A)
12083 COL (A)
04108 ALB (A)
NCPPB382 UK (13C)
04100 SCH (65C)
0748 ONE (65C)
97
04101 SCH (65C)
13109 SUF (13C)
13115 SUF (13C)
99
0785 ALB (65C)
11015 ALB (65C)
0580 WAS (81C)
0687 WAS (81C)
0691 WAS (81C)
95
0692 WAS (81C)
0581 ALB (B)
0767 ONE (81C)
0310 WAS (81C)
0426 WAS (81C)
04106 SCH (D)
13091 OND (65C)
13054 GEN (65C)
13084 WNY (65C)
69
0779 ONE (65C)
0780 ONE (65C)
99
0572 ULT (13C)
0582 ALB (13C)
0651 ULT (13C)
0654 ULT (13C)
99
0676 ORG (13C)
0784 REN (65C)
99
12084 REN (65C)
0690 REN (13C)
64
104r REN (65C)
08223 ULT (81C)
98
08224 ULT (81C)
0763 ORG (81C)
93
13129 SUF (81C)
0775 ONE (81C)
12069 CLT (65C)
13090 CAY (D)
83
13134 WAS (D)
13048 YAT (A)
100
0317 ONT (A)
NCPPB2137
0.01

Figure 1.3: Maximum-likelihood phylogenetic tree for the concatenated housekeeping genes – *dnaA*, *gyrB*, *ligA*, *kdpA*, and *sdhA* – for New York *Clavibacter michiganensis* subsp. *michiganensis* isolates (n=51), reference *C. michiganensis* subsp. *michiganensis* strain NCPPB382, and reference *C. michiganensis* subsp. *sepedonicus* strain NCPPB2137. Isolate numbers, county symbols, and BOX-A1R patterns are indicated. Bootstrap values are shown at the nodes if greater than 50%. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site (Tamura et al. 2011).

Only four of the isolates – 0779, 0780, 13054, and 13091 – would consistently group together in the ML phylogenetic trees, but 04106 and 0784 would not (Figures 1.3 and 1.4). The indel was treated as a single polymorphic site.

All MLSA gene sequences were deposited in GenBank [accession no.: *dnaA* (KJ723713 - KJ723763), *gyrB* (KJ723764 - KJ723814), *kdpA* (KJ723815 - KJ723865), *ligA* (KJ723866 - KJ723916), *sdhA* (KJ723917 - KJ723967), *tomA* (KJ723968 - KJ724018), *nagA* (KJ724019 - KJ724068), *celA* (KJ724069 - KJ724116)].

Comparing the genetic diversity of *C. michiganensis* subsp. *michiganensis* isolates from New York and Serbia. Twenty-three concatenated sequences – *ligA*, *kdpA*, *sdhA*, *tomA* – were aligned including seven Serbian and sixteen New York *C. michiganensis* subsp. *michiganensis* isolates, each representing a unique haplotype (Table 1.5). In comparison to the reference strain NCPPB382, Serbian isolate P520 had the most polymorphic sites with 44 differences, and Serbian isolate P137 had the fewest polymorphic sites at only 11 (Table 1.5). An AMOVA analysis demonstrated that the two populations were not significantly different ($P = 0.276$), with 98% of the genetic variation occurring within populations and only 2% of the genetic variation occurring between populations. Both Serbian and New York isolates were distributed throughout a ML phylogenetic tree with no distinct population clusters (Figure 1.5). Several New York and Serbian isolates demonstrated high levels of similarity, most notably 0317 and

P121.

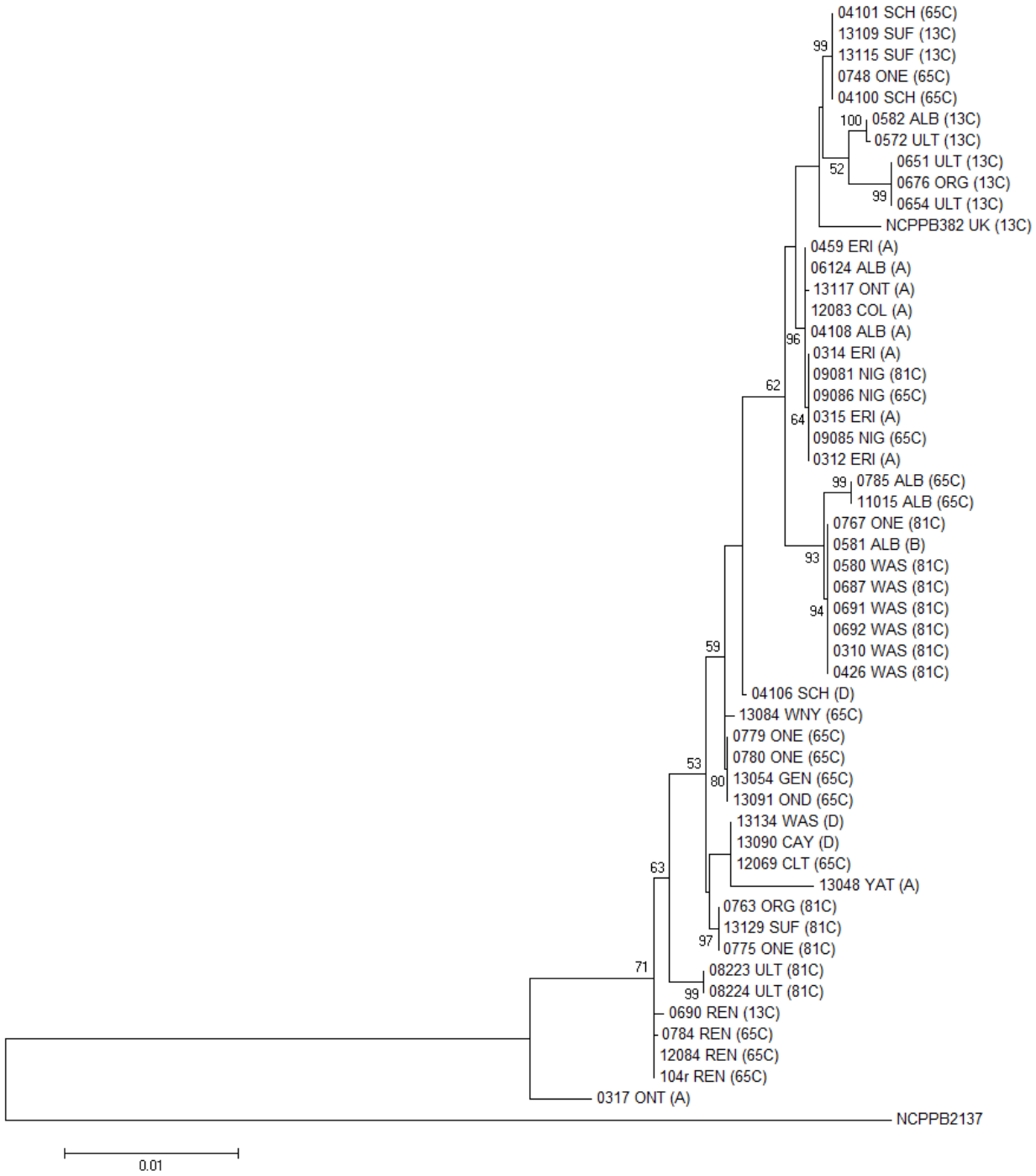

Figure 1.4: Maximum-likelihood phylogenetic tree for the concatenated housekeeping and putative virulence genes – *dnaA*, *gyrB*, *ligA*, *kdpA*, *sdhA*, *tomA*, *nagA*, and *celA* – for New York *Clavibacter michiganensis* subsp. *michiganensis* isolates (n=51), reference *C. michiganensis* subsp. *michiganensis* strain NCPPB382, and reference *C. michiganensis* subsp. *sepedonicus* strain NCPPB2137. Isolate numbers, county symbols, and BOX-A1R patterns are indicated.

Bootstrap values are shown at the nodes if greater than 50%. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site (Tamura et al. 2011).

Table 1.5. Sequence variation in concatenated genes – *ligA*, *kdpA*, *sdhA*, *tomA* – for *Clavibacter michiganensis* subsp. *michiganensis* isolates from New York and Serbia, in respect to reference strain NCPPB382

Origin	Isolate[a]	No. of polymorphic sites[b]	Ave. No. of SNPs (Standard Deviation)
New York	0314	20	26.6 (±8.5)
	06124	19	
	13117	20	
	11015	30	
	0767	27	
	0572	20	
	0582	19	
	04101	13	
	04106	24	
	0651	21	
	0779	27	
	13129	35	
	12069	32	
	0317	36	
	08223	42	
	0690	40	
Serbia	P10	27	28.6 (±10.8)
	P64	24	
	P70	27	
	P121	40	
	P137	11	
	P140	27	
	P520	44	

[a] Selected isolates represent unique haplotypes observed in either New York or Serbia. Unique New York haplotypes from the concatenated housekeeping genes (n=16) and unique Serbian isolates from each of the seven MLST groups (n= 7) were selected (Milijašević-Marčić et al. 2012).

[b] Number of polymorphic sites was determined by comparing sequences to the reference *Clavibacter michiganensis* subsp. *michiganensis* strain NCPPB382.

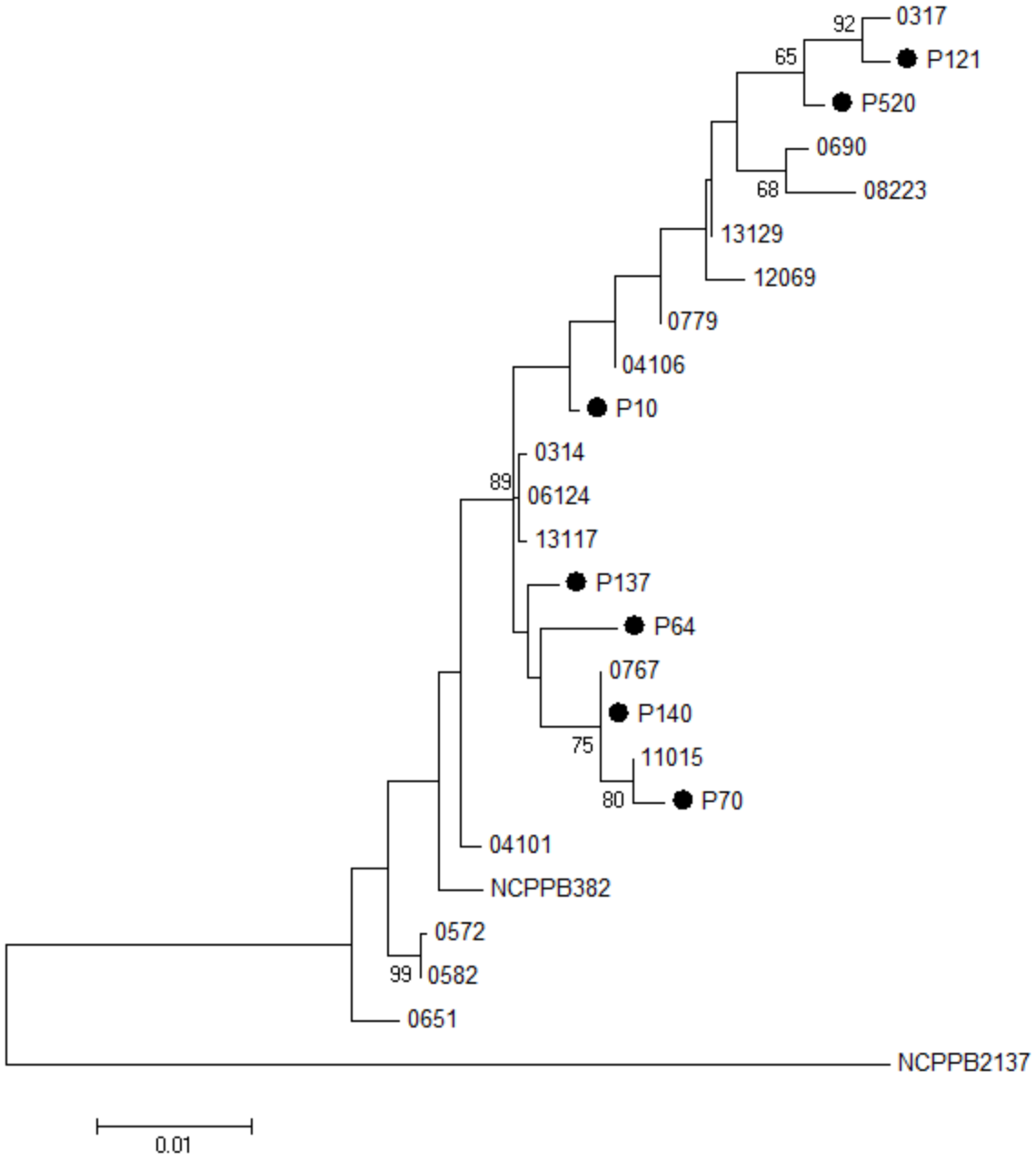

Figure 1.5: Maximum-likelihood phylogenetic tree for the concatenated genes – *ligA*, *kdpA*, *sdhA*, and *tomA* – for reference *C. michiganensis* subsp. *sepedonicus* strain NCPPB2137 and *Clavibacter michiganensis* subsp. *michiganensis* isolates from New York, United Kingdom, and Serbia (n=24). Isolates from Serbia are designated with black dots (Milijašević-Marčić et al. 2012), while NCPPB382 is from the United Kingdom. Bootstrap values are shown at the nodes if greater than 50%. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site (Tamura et al. 2011).

DISCUSSION

New York *C. michiganensis* subsp. *michiganensis* isolates collected from diseased plants over an eleven-year period were highly diverse. The majority of isolates were pathogenic, but differences in disease incidence were observed. However, the association of virulence with putative virulence genes and/or plasmids was not always absolute. Several isolates with plasmids and all six putative virulence genes produced symptoms identical to isolates lacking some virulence genes and plasmids. Similarly, plasmid profiles were unique, diverse, and different from both Serbian and Israeli *C. michiganensis* subsp. *michiganensis* populations (Milijašević-Marčić et al. 2012; Kleitman et al. 2008).

All six BOX-A1R patterns were present in New York with the 'C' – 13C, 65C, 81C – and 'A' types being the most prevent, while 'B' and 'D' were rare. Similar observations were observed in Michigan where the most prevalent BOX-A1R pattern was 'C', with 'A', 'B' and 'D' being observed in a limited number of regions (Quesada-Ocampo et al. 2012). On the contrary, Japanese and Israeli *C. michiganensis* subsp. *michiganensis* populations were predominately 'B' and 'A', with types 'C' and 'D' being less abundant in Japan and rare in Israel (Kleitman et al. 2008; Kawaguchi et al. 2010). Unfortunately, the benefits of utilizing repetitive-PCR as a method of determining population structure, whether within fields or regions, is limited because of its low discriminatory power, subjectiveness, and low inter-lab reproducibility. Therefore, we used an MLSA scheme with a high discriminatory power (0.946), which allowed for a more detailed interpretation of the diversity and potential movement of *C. michiganensis* subsp. *michiganensis* isolates.

The MLSA assay revealed that putative virulence genes were much more conserved than housekeeping genes in New York isolates, with *chpE*, *pat-1*, and *phpA* having no polymorphic

sites in the sequenced regions, while *nagA* only had two single nucleotide polymorphisms (SNPs). Similar results were observed with the Serbian *C. michiganensis* subsp. *michiganensis* isolates, where no SNPs were present within the related serine protease gene *chpC* (Milijašević-Marčić et al. 2012). Conversely, a 2012 Michigan *C. michiganensis* subsp. *michiganensis* survey found *pat-1* to be the most variable gene with 45 SNPs identified in the 96 isolates sampled, even though the same region was sequenced in the present study (Quesada-Ocampo et al. 2012). Unfortunately, *pat-1* and *celA* were the only shared genes between the current study and the Michigan survey so population diversity comparisons could not be made (Quesada-Ocampo et al. 2012).

Only one isolate in this study, 09085, lacked *chpE* or *nagA* and was also greatly reduced in virulence. While we obviously cannot conclude that the losses of these two genes are responsible for reduction in virulence, the potential role of the gene products in pathogenicity is interesting. Both ChpE and NagA are produced *in planta*, contain signal peptides, and are upregulated >18 or >20 fold, respectively, during infection-mimicking conditions (Savidor et al. 2012). The role of *nagA* is unknown, but β-N-acetylglucosaminidase (or the N-acetylglucosamine metabolic family) has been associated with virulence in a variety of bacterial and eukaryotic pathogens including: *Xanthomonas campestris* pv. *campestris*, *Candida albicans*, *Streptococcus pneumonia*, and *Vibrio cholerae* (Qian et al. 2005; Cannon et al. 1994; Kumar et al. 2000; Jermyn and Boyd 2002; Bateman et al. 2005). Within *Vibrio cholerae*, genes involved in the utilization of N-acetylglucosamine (*nan-nag* region) were found to reside in the VPI-2 pathogenicity island, similarly to *nagA* residing within the pathogenicity island of *C. michiganensis* subsp. *michiganensis* (Jermyn and Boyd 2002). The functions of these N-acetylglucosamine catabolic genes, especially β-N-acetylglucosaminidase, remain speculative;

however, in *Streptococcus pneumonia* they are hypothesized to provide an adhesive function in biofilm formations (Bateman et al. 2005).

The *chpE* gene is interesting because it was found to be the most highly upregulated putative serine protease within the *chp* gene cassette – *chpA*-*chpG* – under infection-mimicking conditions (Savidor et al. 2012). Three of the *chp* genes – *chpA*, *chpB*, and *chpD* – are pseudogenes and while the function of the remaining four Chp peptides is unknown, they are hypothesized to facilitate plant colonization and nutrient acquisition (Stork et al. 2008).

Out of the three plasmid-associated virulence genes (*phpA*, *celA*, *pat-1*), fourteen isolates lacked at least one of the three, with *phpA* being the most common gene to be lost (n=13) and *celA* was the least likely to be lost (n=3). A large proportion of the isolates that lost the *phpA* gene were clustered in the 'A' BOX-A1R PCR pattern (n=8), yet no correlation with virulence and loss of *phpA* was observed (Table 1.3). Interestingly, *C. michiganensis* subsp. *michiganensis* 0317 lacked both *celA* and *phpA*, but was pathogenic on tomato (disease incidence of 6/6), while the two remaining isolates that lacked *celA* – 12084 and 10-4R – had a disease incidence of only 4/6. This further demonstrates the variability in virulence observed among a diverse population of field isolates, and the hypothesized importance of putative virulence genes. Similar observations were reported in a study by Yasuhara-Bell et al. (2013), who found that field isolates remained pathogenic even though the putative virulence genes, *chpC* or *ppaA*, were absent (Yasuhara-Bell et al. 2013).

Six New York *C. michiganensis* subsp. *michiganensis* isolates possessed a 20 bp insertion upstream of the *celA* start codon, but no clear association with disease incidence was observed during the greenhouse pathogenicity assays. The presence of this insertion in only six isolates observed over a ten year period (2004-2013) in disparate regions of New York is

intriguing, but highlights the dynamic nature of virulence genes within populations of *C. michiganensis* subsp. *michiganensis.* Virulence genes within conjugative plasmids, such as the case with pCM1 and pCM2 in NCPPB382, could be rapidly dispersed throughout a population and transfer novel genetic variations that could be advantageous under specific environmental conditions (Chalupowicz et al. 2010).

The ephemeral nature of virulence within field populations of *C. michiganensis* subsp. *michiganensis* can be difficult to study since the isolation of avirulent isolates from diseased plants is relatively rare (Eichenlaub and Gartemann 2011). In our study however, several isolates (isolated from the same farm over a seven year period) had differences in putative virulence genes, but the highly polymorphic housekeeping genes remained conserved. For example, 0690 was isolated from a farm for the first time in Rensselaer County during 2006 and the same haplotype (based on housekeeping genes) was reisolated from the farm during a 2007 outbreak (0784). The grower took appropriate measures and successfully suppressed bacterial canker outbreaks from 2008-2011; unfortunately, the same haplotype (based on housekeeping genes) was again reisolated in a 2012 outbreak (12084). Unexpectedly, an irrigation water survey detected viable colonies of the same haplotype (based on housekeeping genes) of *C. michiganensis* subsp. *michiganensis* in creek irrigation water from the same county, but different cities (approximately 24 miles away) during 2010 (10-4R) (Jones 2014). Therefore, it appears that the isolate was maintained in or around the surrounding area for several years without any noticeable (or reported) outbreaks occurring, possibly due to improper sanitation measures.

The initial 2006 (0690) and 2007 (0784) *C. michiganensis* subsp. *michiganensis* isolates each possessed the six putative virulence genes with the same plasmid profiles, but five SNPs were detected within the *celA* gene, including a 20 bp insertion upstream of the promoter in

0784; thereby, differentiating 0690 and 0784. On the contrary, 10-4R and 12084 lost their plasmids (profile P8), with 10-4R losing *pat-1*, *celA*, and *phpA* and 12084 only losing *pat-1* and *celA* based on our PCR analysis. All of the isolates were pathogenic based on greenhouse studies, but 10-4R and 12084 were weak pathogens.

The reemergence of an identical haplotype over an extended period of time highlights the difficulty in properly managing bacterial canker outbreaks. This trend has been observed several times including: isolates 0785 and 11015 (identical haplotype and collected from the same farm four years later), isolates 0317 and 13048 (identical haplotype based on housekeeping genes, but isolated from farms in adjoining counties eleven years apart), and isolates 0310, 0426, 0580, 0687, 0691, and 0692 (identical haplotype collected from the same grower over a four year period). Alternatively, novel haplotypes – 0310, 0317, 04016, 0582, 0690, 0785, 08223, 13084, 13117 – that were isolated from farms with no previous history of *C. michiganensis* subsp. *michiganensis*, could have been introduced from infected seed or transplants. Having the ability of utilizing MLSA to identify strains (both novel and old) could assist growers by demonstrating that they could be maintaining the same population of *C. michiganensis* subsp. *michiganensis* on their farms due to improper sanitation methods or from an introduction event.

Besides looking at the genetic diversity at a statewide level, we wanted to better understand global *C. michiganensis* subsp. *michiganensis* populations, and a recent study from Serbia enabled such a comparison due to the overlap in primer sets (Milijašević-Marčić et al. 2012). Interestingly, 98% of the genetic variation observed between New York and Serbian isolates occurred within populations and only 2% of the genetic variation occurred between populations. This low level of genetic variation between two geographically separated populations could suggest both populations shared a common origin. Additionally, the lack of

any significant divergence occurring between the two populations could further demonstrate a recent introduction event. Accordingly, Milijašević-Marčić et al. (2012) noted that bacterial canker outbreaks have gone unreported for approximately 50 years in Serbia, until large greenhouse outbreaks occurred around 2006-2008 throughout the country. Further insight into the movement and population biology of *C. michiganensis* subsp. *michiganensis* could be formulated with additional populations being examined and compared.

The data presented here demonstrates that the New York population of *C. michiganensis* subsp. *michiganensis* is highly diverse with 21 haplotypes observed over an eleven-year period. Several of the isolates are reoccurring, but many are novel and have been observed on farms with no previous history of bacterial canker of tomato. Our MLSA scheme, comprised of five housekeeping genes and three putative virulence genes, resulted in a discriminatory power of 0.946. Interestingly, a recent phylogenetic analysis (MLSA) of *C. michiganensis* had a discriminatory power of 0.972 (with 48 haplotypes) for six housekeeping genes (Jacques et al. 2012). That analysis included all five *C. michiganensis* subspecies (plus unknown *Clavibacter*-like saprophytes) with 69 globally collected *C. michiganensis* subsp. *michiganensis* isolates from over a half a century of collections (Jacques et al. 2012). It was interesting to observe a similar amount of diversity when just New York *C. michiganensis* subsp. *michiganensis* isolates from an eleven year period were analyzed. Moreover, a multilocus variable-number-tandem-repeats analysis from 56 European *C. michiganensis* subsp. *michiganensis* isolates collected from over a half a century resulted in a discriminatory power of just 0.800 (Zaluga et al. 2013).

Overall, it becomes ever more important for the consistent use of the most polymorphic genes (as a common standard) to be set for future *C. michiganensis* subsp. *michiganensis* population studies. Hopefully, additional researchers will characterize their *C. michiganensis*

subsp. *michiganensis* populations using an MLSA approach, in which comprehensive epidemiological patterns may be observed that will better illuminate the population biology of this devastating plant pathogen.

ACKNOWLEDGEMENTS

This research was supported by grant no. IS-4403-11C from the United States-Israel Binational Agricultural Research and Development (BARD) Fund. Support for M. Tancos was additionally provided by a fellowship from Cornell University College of Agriculture and Life Sciences and the National Science Foundation-Graduate Research Fellowships Program (GRFP) grant no. DGE-1144153. Special thanks to Kiersten Bekoscke and members of the Smart lab for assisting with various experiments.

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CHAPTER 2

TOMATO FRUIT AND SEED COLONIZATION BY *CLAVIBACTER MICHIGANENSIS* SUBSP. *MICHIGANENSIS* THROUGH EXTERNAL AND INTERNAL ROUTES[2]

ABSTRACT

The Gram-positive bacterium *Clavibacter michiganensis* subsp. *michiganensis*, causal agent of bacterial wilt and canker of tomato, is an economically devastating pathogen that inflicts considerable damage throughout all major tomato producing regions. Annual outbreaks continue to occur in New York where *C. michiganensis* subsp. *michiganensis* spreads by infected transplants, trellising stakes, tools and/or soil. Globally, new outbreaks can be accompanied by the introduction of contaminated seed stock; but the route of seed infection, especially the role of fruit lesions, remains undefined. In order to investigate the modes of seed infection, New York *C. michiganensis* subsp. *michiganensis* field strains were stably transformed with a gene encoding eGFP. A constitutively eGFP-expressing virulent *C. michiganensis* subsp. *michiganensis* isolate, GCMM-22, was used to demonstrate that *C. michiganensis* subsp. *michiganensis* could not only access seeds systemically through the xylem, but also externally through tomato fruit lesions, which harbored high intra-and intercellular populations. Active

[2] Reprinted from Tancos, M.A., Chalupowicz, L., Barash, I., Manulis-Sasson, S., and Smart, C.D. 2013. Tomato fruit and seed colonization by *Clavibacter michiganensis* subsp. *michiganensis* through external and internal routes. *Applied and Environmental Microbiology* 79:6948-6957.

movement and expansion of bacteria into the fruit mesocarp and nearby xylem vessels followed, once the fruit began to ripen. These results highlight the ability of *C. michiganensis* subsp. *michiganensis* to invade tomato fruit and seed through multiple entry routes.

INTRODUCTION

Seed-disseminated phytopathogens exemplify the adaptive nature of parasites by not only gaining access to seed, surviving seed treatment processes, and colonizing emergent seedlings, but also by attaining global distribution. *Clavibacter michiganensis* subsp. *michiganensis*, the causal agent of bacterial wilt and canker of tomato, continues to cause epidemics throughout all major tomato-producing regions, and insuring healthy seed stock remains a top priority (de León et al. 2011). *C. michiganensis* subsp. *michiganensis* was initially isolated from Michigan (USA) in 1909 and rapidly spread to nearby states, including New York (Bryan 1930). Unfortunately, annual outbreaks of *C. michiganensis* subsp. *michiganensis* continue to occur in New York, which has over 2,900 acres of fresh market tomato valued at 47.1 million dollars, representing the 4th most valuable vegetable in the state (USDA 2013). Besides its impact on agriculture in the USA, *C. michiganensis* subsp. *michiganensis* is listed as quarantine pest for Europe, Asia, the Caribbean, and Africa (de León et al. 2011).

The worldwide dissemination of this Gram-positive bacterium is facilitated by contaminated seed stock, in which 1 infected seed in 10,000 is capable of initiating an epidemic (de León et al. 2011; Bryan 1930; Chang et al. 1991). Currently there are no resistant cultivars and few chemical controls are effective. Thus, sanitary practices are important for disease control (Werner et al. 2002; Sen et al. 2013). *C. michiganensis* subsp. *michiganensis* survives in soil, on trellising stakes, greenhouse benches and tools for months to years due to its ability to

tolerate desiccation and cold temperatures (Chang et al. 1991; Werner et al. 2002; Bryan 1930). Small bacterial populations can rapidly increase during commercial transplant production by water splash and/or equipment (Carlton et al. 1998). In addition, outbreaks frequently remain un-noticed for extended periods of time since latent infections and symptomless seedlings are relatively common (Gitaitis et al. 1991; Werner et al. 2002; Chang et al. 1991).

The pathogenic nature of *C. michiganensis* subsp. *michiganensis* strain NCPPB382 is derived from its many putative serine proteases and cell-wall-degrading enzymes, which are encoded by two plasmids (pCM1 & pCM2) and a pathogenicity island located on the chromosome (Gartemann et al. 2008; Eichenlaub and Gartemann 2011; Balaji et al. 2008). *C. michiganensis* subsp. *michiganensis* enters the tomato epiphytically through natural openings and wounds or from infected seed (Carlton et al. 1998; Bryan 1930; de León et al. 2011). Once inside a plant, the bacterium multiplies in the xylem vessels, forming extensive biofilm-like structures, which aid in pathogen colonization and movement (Chalupowicz et al. 2012). Systemic infection with high populations of $>10^8$ CFU/g, lead to the characteristic wilting, stem canker, and vascular discoloration (Bryan 1930; Gartemann et al. 2003). Additionally, the pathogen can ooze from cankers and hydathodes, and in combination with rain and wind, the pathogen spreads to distal leaves, fruit, and surrounding plants (Bryan 1930; Sharabani et al. 2013). Bacteria present on the fruit surface can cause 'bird's-eye' lesions that consist of small tan dots with white halos (Bryan 1930; Medina-Mora et al. 2001).

To better understand the movement of *C. michiganensis* subsp. *michiganensis in planta*, strains have been previously transformed to express either the *lux* operon or the eGFP gene (Xu et al. 2010; Chalupowicz et al. 2012). However, the initial eGFP transformation was transient and only remained stable for approximately one month, whereas bioluminescent transformation

inhibits the ability to track individual bacterial cells at low populations (Chalupowicz et al. 2012). In this study, New York field strains were stably transformed with eGFP to better understand the natural routes of infection that could lead to contaminated tomato seed.

Phytopathogens can access their host's seed systemically through the vasculature or externally by penetrating the ovary wall or floral parts (Agarwal and Sinclair 1997; Singh and Mathur 2004). Since *C. michiganensis* subsp. *michiganensis*, is a systemic pathogen it is hypothesized that it accesses the developing seeds via the host vascular system (de León et al. 2011). One of the earliest studies on bacterial canker looked at the systemic movement of the pathogen to the seed, however, no detailed histopathological studies have clearly demonstrated this natural route of seed infection (Bryan 1930; Singh and Mathur 2004); and the significance of bird's-eye lesions in fruit colonization remain unstudied. Our working hypothesis was that seed contamination by *C. michiganensis* subsp. *michiganensis* could occur through both systemic and external fruit inoculations. The objectives of this study were to test the ability of *C. michiganensis* subsp. *michiganensis* to colonize developing tomato fruit and seed either (i) systemically through the xylem and/or (ii) externally by entering fruit through lesions on the exocarp.

MATERIALS & METHODS

Bacterial strains and growth conditions. The *C. michiganensis* subsp. *michiganensis* strains utilized in the present study were New York State field strains collected from multiple bacterial canker outbreaks (Table 2.1). The four individual field strains were chosen based on the highly aggressive nature observed on tomato. Depending on the assay, *C. michiganensis* subsp. *michiganensis* strains were incubated for 3-6 days at 27°C in Luria-Bertani (Miller 1972),

SB (Kirchner et al. 2001; Stork et al. 2008), or D2ANX media (Hadas et al. 2005). When required, LB and SB media were supplemented with the antibiotic chloramphenicol (10µg/ml) (Fischer Scientific; Pittsburgh, PA).

Table 2.1: *Clavibacter michiganensis* subsp. *michiganensis* strains used in this study

Strain No.	County of Origin	Year collected	Voltage (kV/cm)[a]	Transformation Efficiency[b]	Reference
04101-FS	Schoharie, NY	2004	12.5	1 (2)	(Balaji and Smart 2012; Balaji et al. 2011)
0690-FS	Rensselaer, NY	2006	12.5	16 (18)	This study
0767-FS	Oneida, NY	2007	7.5	16 (27)	This study
11015-FS	Albany, NY	2011	12.5	3 (3)	This study

[a] Voltages shown are the electroporation settings used to transform field strains.
[b] The optimized transformation efficiency is shown as transformants/µg of vector DNA. Numbers in parentheses indicate total number of eGFP-transformants collected.

Construction of the plasmid pKGT-GFP. The entire coding region of e*gfp*, with its upstream DNA fragment derived from the *C. michiganensis* subsp. *michiganensis* bacteriophage CMP1, was amplified with the primers GFP-SpeI-fow (TTGAACCACTAGTCAGTACTG) and GFP-SpeI-rev (ACGGGCACTAGTAGTGAG) using pK2-22 (the plasmid expressing eGFP), as a template (Figure 2.1) (Chalupowicz et al. 2012). The generated 1305-bp PCR product was cloned into pGEM-T easy vector (Promega Corp, Madison, WI) to yield pGeStbGFP. The artificially created *spe*I restriction sites included in the PCR primers were used to digest total plasmid DNA and generate a 1294-bp *speI* DNA fragment. The latter was cloned into the unique *speI* restriction site of the Tn*1409* transposon vector pKGT452Cβ (Kirchner et al. 2001) to yield

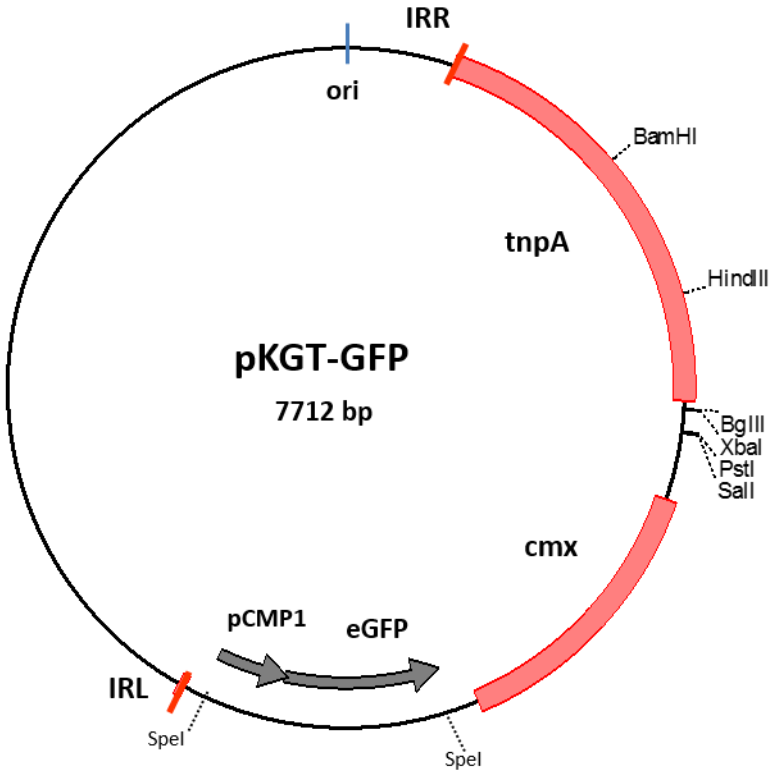

Figure 2.1. Physical map of the plasmid, pKGT-GFP, expressing the green fluorescent protein gene (*egfp*). A PCR-generated DNA fragment carrying the *egfp* (eGFP) gene and a sequence derived from *Cmm* bacteriophage CMP1 (pCMP1) was inserted into the unique *speI* restriction site of the Tn*1409* transposon vector pKGT452Cβ. *cmx* = chloramphenicol resistance gene, ori = origin of replication of the vector pUC13, *tnpA* = transposase IS *1409,* IRL = left inverted repeat, IRR = right inverted repeat.

pKGT-GFP. In this plasmid, *spe*I is located downstream of the IRL (left inverted repeat) and 103 bp upstream of the chloramphenicol exporter gene, *cmx.* The pKGT-GFP was transferred into *E.coli* JM109 and recombinant colonies with the green fluorescence phenotype were selected from LB agar plates containing chloramphenicol (10µg/ml). Orientation of the eGFP insertion was verified with PCR primers, GFP-SpeI-fow and pKGT-Cmx-rev (AACACGAGAAGGCAAACT).

Transformation and isolation of eGFP-labeled *Clavibacter michiganensis* subsp. *michiganensis*. DNA of pKGT-GFP was extracted from *E. coli* JM109 with E.Z.N.A Fastfilter

plasmid midi kit (Omega Bio-Tek; Norcross, GA) and subsequently electroporated into *C. michiganensis s*ubsp. *michiganensis* field strains. Competent cells were prepared as previously described by Stork et al. (Stork et al. 2008). Electroporation was performed with 50µl of competent cells and 1µg of the purified eGFP vector using the Bio-Rad Gene Pulser Xcell electroporation system (Hercules, CA) with the following settings: 0.2-cm electroporation cuvettes, 7.5-12.5 kV/cm (Table 2.1), 25µF, and 600 Ohm with a time constant between 12 and 16 msec. Increased transformation rate was observed when 2 pulses were applied with a 20 second interval between pulses (Laine et al. 1996). Cells were immediately mixed into SB medium and regenerated for 2 hours at 27°C. Cells were spread onto SB agar plates containing chloramphenicol and incubated for 5-7 days (Chalupowicz et al. 2012).

Fifty eGFP-mutants, derived from the 4 different field strains, were isolated and grown for 48 hours in LB broth amended with chloramphenicol at 27°C. Triplicates of 100 µl of culture (optical density at 600 nm = 0.5) were screened for fluorescence emission using a 96-well black microtiter plate (Fisher Scientific; Pittsburgh, PA) and the Synergy 2 BioTek microplate reader (Winooski, VT) (Chalupowicz et al. 2012). Fluorescence units (FUs) were normalized by subtracting the background florescence from control wells of wild-type *C. michiganensis* subsp. *michiganensis* grown in LB broth at the same concentration. EGFP-isolates with >7000 FU, at a sensitivity level set to 70, were further analyzed. The experiment was repeated three times for a total of nine fluorescence emission readings.

Pathogenicity assays. Tomato seedlings (*Solanum lycopersicum),* cultivar Mountain Fresh Plus, were grown in a Cornell potting mix (composed of peat, perlite, and vermiculite in a 4:1:1 ratio) with a 14-hr light/ 10-hr dark photoperiod in the greenhouse. The eGFP-*C. michiganensis* subsp. *michiganensis* isolates and parental field strains were grown for 48-72 hours in LB medium

(10µg/ml of chloramphenicol were added to eGFP-isolates) at 27°C, diluted to 10^8 CFU/ml and inoculated onto approximately 2 week old tomato seedlings (n=6/isolate) by the cotyledon clipping method (Xu et al. 2010). The wild-type field stains and sterile water were used as positive and negative controls, respectively. After the first week, tomato plants were screened daily for characteristic wilting and chlorosis. Ratings continued until all plants died or until 24 days post inoculation. Disease incidence was used to compare the eGFP-isolates to their respective field strains, in order to determine if isolates remained virulent following transformation. Five tomato plants comprised a replicate, and each treatment was replicated three times. The mean area under the disease progress curve (AUDPC) was calculated from disease incidence (Madden et al. 2007).

Approximately ten days following inoculation, one of the six tomato seedlings from each treatment was harvested to quantify *in planta* bacterial populations. A 1 cm stem section above the inoculation site was aseptically removed from the harvested plants and homogenized in 400 µl of sterile 10mM $MgCl_2$ via the Retsch MM400 tissuelyser (Newton, PA). Following complete homogenization, 600 µl of sterile 10mM $MgCl_2$ was added for a final volume of 1 ml (Balaji and Smart 2012). The solution was spun down at 400 x g for 3 minutes (Xu et al. 2010). Serial dilutions were performed with the supernatant and plated onto either LB with chloramphenicol or D2ANX (for wild-type field strains and negative controls) and incubated for 4-6 days at 27°C. The *C. michiganensis* subsp. *michiganensis* populations (CFU/g of tissue) were calculated by: weight of the sample x volume plated x number of colonies x dilution factor. Significant differences among treatments for AUDPC and *in planta* populations were tested with ANOVA ($P<0.05$) followed by Dunnett and Tukey's Studentized Range posttests ($P=0.05$) using SAS v. 4.3 (SAS Institute, Cary, NC). The experiment was repeated three times.

External inoculation of fruit. Twenty Mountain Fresh Plus tomatoes were grown as described above, and transplanted into one gallon pots at 3 weeks of age so plants would mature and flower. The flowers were artificially pollinated with a hand-held vibrating tomato pollinator, model 5E846, (Hydro-Gardens Worldwide, Inc., Colorado Springs, CO) multiple times per week. The eGFP isolate, GCMM-22, was prepared by growing the isolate at 27°C and shaking at 140 rpm for 48-72 hours in liquid LB with chloramphenicol. The bacterial suspension was diluted to a density of 10^8 cells/ml, and was applied to the entire surface of immature green fruit 8-12 mm in diameter (n=50) using a #2 horse-hair paintbrush as previously described (Medina-Mora et al. 2001). A similar number of tomato fruit (n=41) were brushed with sterile distilled water as a negative control. Twenty-one *C. michiganensis* subsp. *michiganensis* inoculated fruit were harvested and aseptically dissected at the green, breaker, and turning stages of fruit development in agreement with the United States Standards for Fresh Tomatoes Color Classification (USDA 1991). Within those 21 fruit, a total of 182 individual lesions were analyzed using confocal microscopy.

Fruit were collected and either dissected and analyzed immediately or stored at 4 °C for no more than a week prior to dissection. Fruit were laterally sectioned first, and then transverse and lateral sections were made of the two halves. Individual lesions and pericarp tissues were targeted for analysis. All sections were hand sliced with sterile double edged razors and visualized using an Olympus BX61 microscope connected to a confocal laser scanning microscope (CLSM) system (Olympus Fluoview FV-300, Melville, NY). An argon laser (488 nm excitation) and a green helium neon laser (543 nm excitation) was used excite the eGFP bacteria and induce plant autofluorescence, respectively (Dunn et al. 2013). A total of 10 lesions from 5 fruit were excised and plated on antibiotic selective media to confirm that *C.*

michiganensis subsp. *michiganensis* cells observed during microscopy were viable (Medina-Mora et al. 2001). Negative control tomatoes were also harvested and analyzed.

Systemic inoculation of fruit. Flowering tomato plants and eGFP-isolate GCMM-22 were both prepared as described above. For this experiment, 25 µl of bacterial suspension (10^8 cells/ml) were injected (using a syringe and a 26.5G needle) into the peduncle truss of 20 inflorescences (Figure 2.2) (Balaji et al. 2008). This was also performed with the negative control plants (n=12) except sterile water was injected. In order to see the effects of inoculation at different stages of fruit development, inflorescences with floral units at various developmental stages (i.e. bud, flower, dead flower/emerging fruit, <1 cm fruit, or > 1cm fruit stages) were inoculated (Figure 2.2). A total of 49 fruit, corresponding to 20 inoculated inflorescences, were harvested at various time points (days-post-inoculation). Fruit were analyzed as described above, but instead of lesions, tissue slices at varying distances below the calyx were screened for eGFP-*C. michiganensis* subsp. *michiganensis*. To confirm the *C. michiganensis* subsp. *michiganensis* that was observed during microscopy was viable, several 0.5 cm pedicel sections and colonized fruit vascular bundles were excised and plated onto antibiotic selective media (Medina-Mora et al. 2001). Negative control tomatoes were also harvested and analyzed.

Individual pedicels on several inflorescences were also inoculated in a similar manner to the peduncle truss injections. Floral units (n=7), at different stages of development, were selected to see the effects of infection in respect to flower/fruit stages (Figure 2.2). Negative controls (n=3) were also performed.

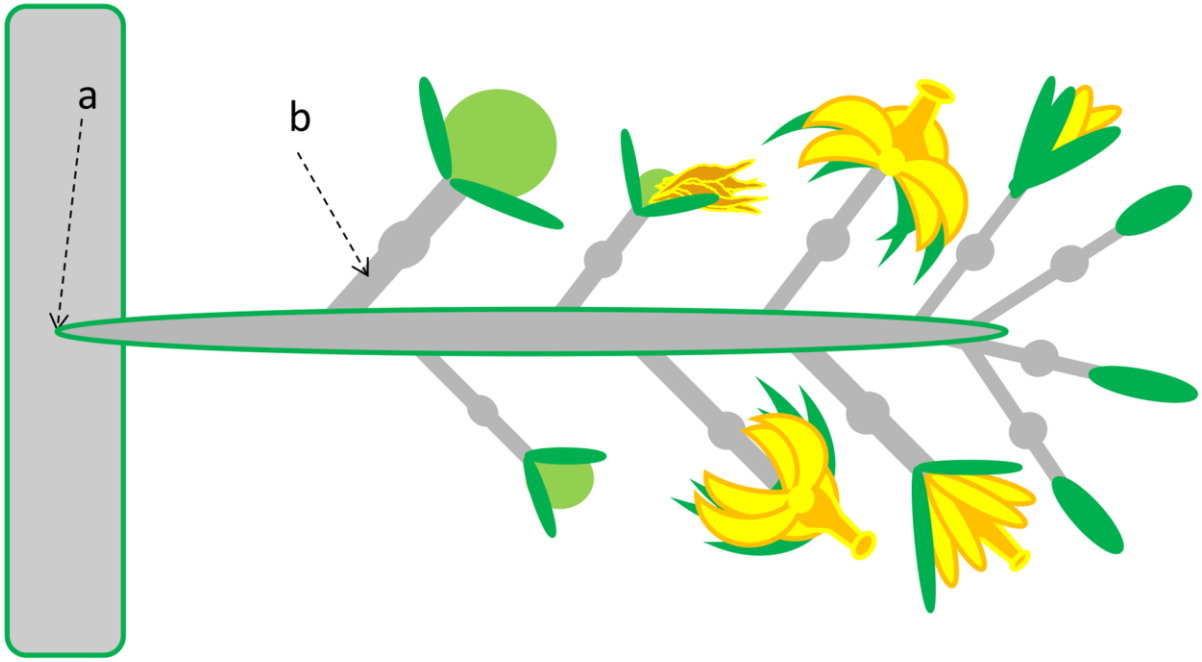

Figure 2.2: Schematic of systemic inoculation points. The inflorescence was injected with *Clavibacter michiganensis* subsp. *michiganensis* at either the stem/peduncle junction (peduncle truss) (a) or the pedicel (b). Several stages of fruit development were commonly present when inoculated: bud, flower, dead flower/emerging fruit, <1 cm fruit, or > 1cm fruit.

RESULTS

Transformation and characterization of eGFP-labeled *C. michiganensis* subsp. *michiganensis* isolates. Transformation efficiency and electroporation voltages varied significantly among field strains; 11015-FS and 04101-FS were very difficult to transform and only a total of 3 and 2 eGFP-isolates were obtained, respectively. However, a total of 27 and 18 eGFP-isolates were obtained from 0767-FS and 0690-FS, respectively (Table 2.1). Between the four wild-type field strains, a total of 50 eGFP-isolates were collected and analyzed for fluorescence intensity. Average emitted fluorescence readings ranged from 58-17,323 fluorescence units (FUs), but only the top fluorescent eGFP-isolates, from each of the four field strains, were further analyzed (Table 2.2). The two 04101-FS eGFP-isolates both had

fluorescence readings around 6,760 FUs, but were not included in the group selected for further analysis because of significantly reduced *in vitro* growth rates.

Table 2.2: Virulence to tomato plants by eGFP isolates and parental *Clavibacter michiganensis* subsp. *michiganensis* strains

Isolate[a]	FU[b]	Disease Incidence[c]	AUDPC[d]	CFU/g in planta[e]
11015-FS	-	14/15	1396.7 a	1.08×10^{10}
GCMM-28	7,059	13/15	1216.7 abc	1.75×10^{10}
0690-FS	-	14/15	1263.3 ab	1.16×10^{10}
GCMM-22	8,252	15/15	1340.0 ab	6.36×10^{9}
GCMM-14	9,585	10/15	966.7 bc	1.02×10^{10}
GCMM-12	17,323	1/15	16.7 d	6.71×10^{9}
GCMM-13	10,446	3/15	50.0 d	7.10×10^{9}
GCMM-24	10,192	11/15	860.0 c	1.19×10^{9}
0767-FS	-	14/15	1223.3 abc	1.38×10^{10}
GCMM-26	14,535	15/15	1543.3 a	2.31×10^{10}
GCMM-44	8,426	14/15	1326.7 ab	2.33×10^{10}
GCMM-27	11,401	6/15	126.7 d	6.37×10^{9}
GCMM-37	9,916	9/15	320.0 d	1.12×10^{10}
Water	-	0/15	0.0 d	0

[a] Wild-type field strains (FS) italicized and bolded, and their corresponding eGFP isolates (GCMM).
[b] FU, fluorescence units. The mean normalized eGFP-fluorescent reading is shown.
[c] Expressed as the number of wilting plants/number of inoculated plants characterized 17-24 days post inoculation.
[d] The mean area under disease progress curve (AUDPC) for disease incidence. Significant differences among treatments were tested with ANOVA ($P<0.05$) followed by Dunnett and Tukey's Studentized Range posttests ($P=0.05$). AUDPC values followed by the same letter are not significantly different.
[e] Mean in planta population sizes recovered from 1 cm stem tissue. No significant differences within field strains were observed when tested with ANOVA ($P<0.05$).

The ten selected eGFP-isolates colonized the tomato stem at similar levels, one cm above the inoculation site, with CFU/g tissue ranging from 10^9 to 10^{10}. Within strains, the differences in the level of colonization were not significantly different based on ANOVA (Table 2.2). In contrast to colonization, disease incidence varied among isolates. The mean AUDPC values for five of the ten eGFP-isolates were significantly less than the wild-type field strains used in the

transformation, while the other five were similar to the wild-type field strains (Table 2.2). With four of the eGFP-isolates, fewer than 10 of the 15 inoculated tomato plants wilted within 24 days post inoculation (Table 2.2). Based upon these findings, eGFP-isolate GCMM-22 was selected for fruit inoculation assays.

Fruit internalization of externally applied eGFP-labeled *C. michiganensis* subsp. *michiganensis.* The tomato fruit pericarp is divided into three layers comprised of the exocarp, mesocarp (location of vascular bundles), and endocarp. Following the external application of GCMM-22, lesions appeared over the entire fruit epidermis of all 50 inoculated fruit in as few as three days and did not increase appreciably in diameter after initial formation (Figure 2.3A). Tomatoes at the green stage ranged in size from 2.7 cm - 5.8 cm in width, corresponding to 7 and 15 DPI, respectively (Table 2.3). All lesions viewed had heavy intra- and intercellular colonization of the exocarp and adjacent mesocarp cells (Figure 2.4). Interestingly, *C. michiganensis* subsp. *michiganensis* appeared to remain constrained to these upper pericarp cell types throughout the entire green stage of fruit development (Table 2.3). However, as the fruit began to ripen, *C. michiganensis* subsp. *michiganensis* could be seen radiating outwards from the lesions in both lateral and basipetal directions at distances > 18-25 parenchyma cells and even penetrating xylem vessels (Figure 2.4B); thereby, leading to the entry of *C. michiganensis* subsp. *michiganensis* into the vascular system.

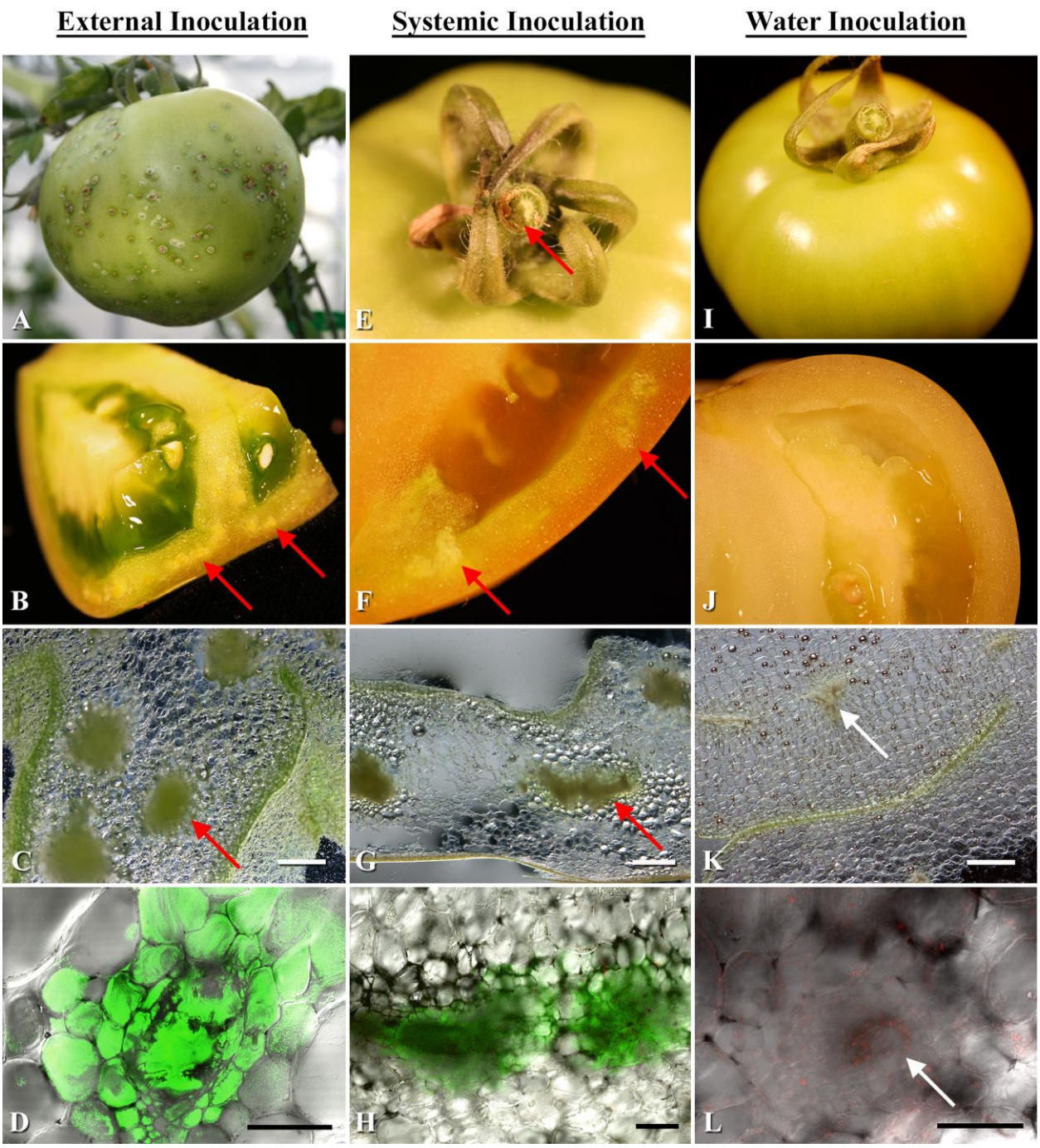

Figure 2.3: *Clavibacter michiganensis* subsp. *michiganensis* (*Cmm*) fruit colonization with different inoculation methods. External inoculation (A-D); (A) Fruit lesions on the tomato exocarp (B-C) Heavy bacterial colonization of pericarp vascular bundles (D) Confocal image of aforementioned vascular bundle with eGFP-labeled *Cmm* colonization. Systemic inoculation (E-H); (E) Fruit with discolored pedicel vasculature (F-G) Heavy bacterial colonization of pericarp vascular bundles (H) Vascular bundles colonized by eGFP-labeled *Cmm*. Water inoculation (I-L); (I) negative control fruit with no fruit lesions or pedicel vasculature discoloration (J-L) negative control pericarp vascular bundles. All confocal microscopy images

(D, H, and L) were generated by merging 3 channels (488 nm, 543 nm, and transmitted light). Red arrows indicate heavy vascular colonization by *Cmm*. White arrows indicate non-colonized vascular bundles. Scale bars: 1 mm in C, G and K; 200 µm in D and H; 100 µm in L.

Table 2.3: *Clavibacter michiganensis* subsp. *michiganensis* movement and colonization of tomato fruit tissue via external fruit inoculation[a]

			No. of lesions with *Cmm* colonization[b]			No. of fruit with *Cmm* colonization[c]		
Tomato stage	DPI	No. of lesions[d]	Exocarp	Upper mesocarp	No. of fruit	Endocarp	Xylem	Seed[e]
Green	5-15	57	57	57	7	0	0	0
Breaker	31-34	56	56	56	7	3	2	1
Turning	32-41	69	69	69	7	3	3	NA
Total	-	182	182	182	21	6	5	1

[a] For the colonization analyses, each fruit was separated into 5 different tissue types: exocarp, mesocarp, xylem (located within mesocarp), endocarp, and seed.
[b] Out of the total number of lesions assessed with confocal microscopy, the numbers noted represent how many lesions were colonized with *Cmm* in either the exocarp or upper mesocarp cells of the pericarp.
[c] The number of fruit colonized by *Cmm* in the endocarp, xylem, or seed was assessed. The distance of endocarp, xylem and seed from external lesions prevents determination of which lesion led to the colonization of these tissues.
[d] Lesions were selected from 7 fruit at each ripening stage and subsequently assessed for colonization with confocal microscopy.
[e] The number of fruit with visible seed colonization is given. NA, not applicable (no seed were analyzed and viewed with the confocal at this late ripening stage due to the inability to effectively slice the seed in the gel matrix of the locular cavity).

Entire locular cavities became colonized, and damaged vascular bundles could be visualized with the naked-eye (Figure 2.3A &B). It appeared that *C. michiganensis* subsp. *michiganensis* would unilaterally colonize fruit, as only individual locular cavities would maintain heavily colonized xylem vessels. Fruit with heavy intravascular growth frequently supported *C. michiganensis* subsp. *michiganensis* populations in the pedicel. Further *in planta* spread was not explored. No pedicels were colonized from fruit lacking *C. michiganensis* subsp. *michiganensis* in fruit xylem vessels, regardless of ripening stage. In total, five fruit (two at the breaker and three at the turning stage) had heavily colonized xylem vessels and vascular bundles

(Table 2.3, Figure 2.3). All cultured lesions and vascular bundles had active bacterial growth. Negative control plants and fruit had no bacterial wilt symptoms and no *C. michiganensis* subsp. *michiganensis* cells were observed during confocal microscopy.

The entry of *C. michiganensis* subsp. *michiganensis* into the fruit xylem, following external inoculation, allowed for the eventual colonization of seed (Table 2.3). Vascular discoloration leading to several developing seed was observed in a fruit at the breaker stage and upon confocal analysis, *C. michiganensis* subsp. *michiganensis* was observed within three seed of that fruit (Figure 2.5). Small numbers of bacterial cells were observed colonizing the seed near the xylem vessel attachment sites and endosperm (Figure 2.5D).

Movement of eGFP-labeled *C. michiganensis* subsp. *michiganensis* inside fruit vasculature from a peduncle truss systemic inoculation. Based on the sequential development of individual floral units on inflorescences, multiple stages of flower/fruit development could be observed on a single inflorescence (Figure 2.2). Therefore, individual floral units were utilized to observe differences in fruit and/or seed infection rates based on the inoculation date. Development of extensive wilting and/or discoloration was not evident on any of the inoculated inflorescences; however, cankers could be seen developing at the site of inoculation, approximately 2-3 weeks post inoculation. No premature fruit abscission or external lesions occurred and infected fruit appeared similar (externally) to the negative controls with similar growth and ripening rates.

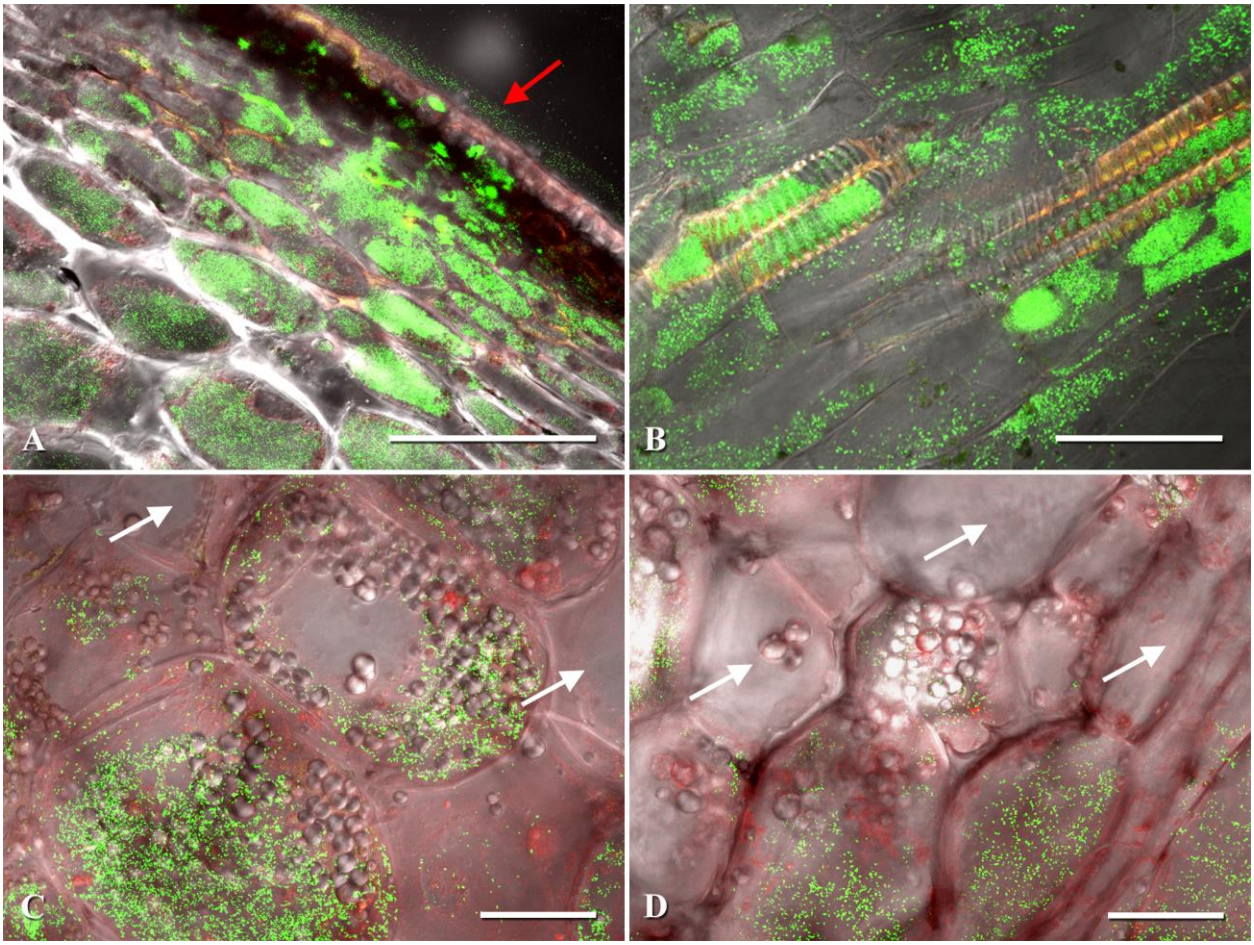

Figure 2.4: Examples of *Clavibacter michiganensis* subsp. *michiganensis* (*Cmm*)-infected pericarp cells of tomato. (A) Pericarp lesion expansion with eGFP-labeled *Cmm* colonizing the exocarp and mesocarp. (B) Fruit xylem and xylem parenchyma colonization by *Cmm.* (C) Intracellular colonization of mesocarp cells by *Cmm.* (D) Intracellular colonization of the endocarp and surrounding parenchyma cells. Confocal microscopy images were generated by merging 3 channels (488 nm, 543 nm, and transmitted light). Red arrow points to the lesion on the fruit surface. White arrows indicate non-colonized fruit cells. Scale bars: 200 µm in A; 50 µm in B, C and D.

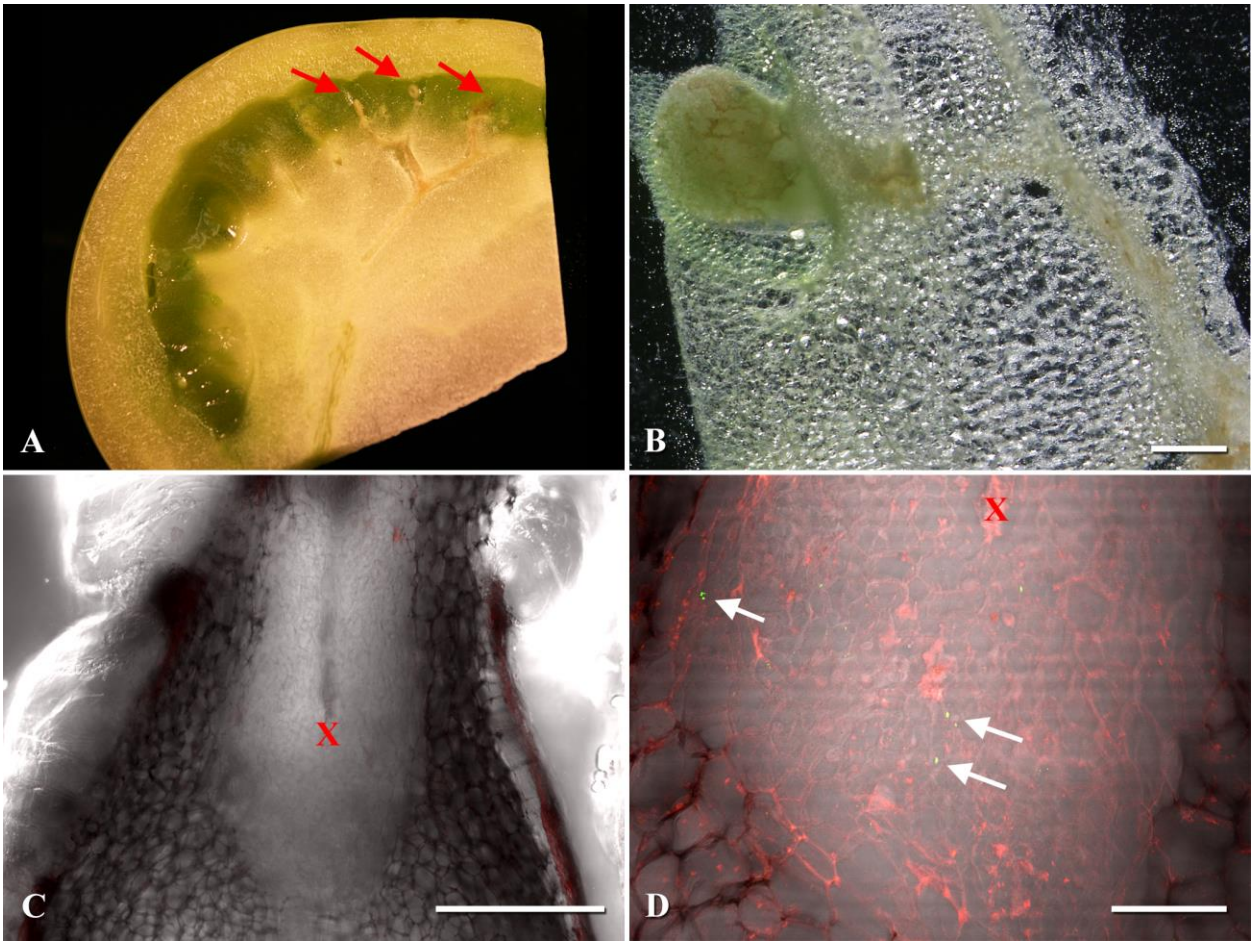

Figure 2.5: External inoculation of tomato fruit with subsequent seed colonization. (A-B) Vascular discoloration leading up to several discolored tomato seed. (C) Thin section of the discolored seed shown in panel B with xylem leading into the seed from the funiculus. (D) Magnified view of seed in panel C with eGFP-labeled *Clavibacter michiganensis* subsp. *michiganensis* (*Cmm*) colonizing the cells surrounding the xylem vasculature. Section of seed corresponding to the same anatomical structure is labeled with a red X. White arrows are pointing to several *Cmm*. Red arrows are pointing to discolored tomato seeds and vasculature. All confocal microscopy images (C and D) were generated by merging 3 channels (488 nm, 543 nm, and transmitted light). Scale bars: 1 mm in B; 200 µm in C; 50 µm in D.

To assess the movement of *C. michiganensis* subsp. *michiganensis* within an inflorescence, fruit were collected at various time points (days post inoculation) and processed at varying distances below the calyx (Table 2.4, Figure 2.3E-H). Not all fruit were infected following inoculation at the peduncle truss, since *C. michiganensis* subsp. *michiganensis* did not effectively colonize the peduncle of 6 of the 20 inoculated inflorescences. Yet, *C.*

michiganensis subsp. *michiganensis* was consistently present ≤ 0.5 cm below the calyx, within the xylem and surrounding xylem parenchyma, in all 25 fruit examined from the 14 inflorescences that were colonized (Table 2.4). In one fruit, vascular bundles at the distal end near the columella, approximately 5 cm below the calyx, became heavily concentrated with *C. michiganensis* subsp. *michiganensis* (Table 2.4, Figure 2.3F-H). Negative control fruit had no vascular discolorations, abnormalities or *C. michiganensis* subsp. *michiganensis* cells.

Table 2.4: *Clavibacter michiganensis* subsp. *michiganensis* movement and colonization of tomato fruit tissue via systemic inoculation at the peduncle truss

DPI[a]	No. of fruit	No. infected fruit	Infection rate (%)	No. of fruit with *Cmm* colonization[b]			
				≤0.5 cm	0.6-1.5 cm	Base of fruit[c]	Seed[d]
21-26	17	9	53	9	7	0	0
27-39	12	7	58	7	4	0	1
40-48	20	9	45	9	5	1	NA
Total	49	25	51	25	16	1	1

[a] Days post inoculation (DPI) when harvested.
[b] Of the infected fruit, the number in which *Cmm* was observed at multiple distances below the calyx or in the seed was assessed.
[c] Vascular bundles colonized by *Cmm* at the base of the fruit represent the maximum distance bacteria travelled from the calyx.
[d] This refers to *Cmm* seen surrounding, internalized, or colonizing the embryo vasculature. NA, not applicable (no seed were analyzed and viewed with the confocal at this late fruit stage due to the inability to effectively slice the seed in the gel matrix of the locular cavity).

Colonization by the pathogen did cause extensive seed discoloration and darkening in some of the more mature fruit. A fruit that was harvested 27 DPI, had heavy xylem colonization and *C. michiganensis* subsp. *michiganensis* could be seen colonizing the xylem vessels of the funiculus, directly leading to the developing seed (Figure 2.6). Unfortunately, the late ripening stages inhibited an extensive microscopic analysis of the collected seed due to the inability to effectively dissect the seed in the highly autofluorescent gel matrix of the locular cavities.

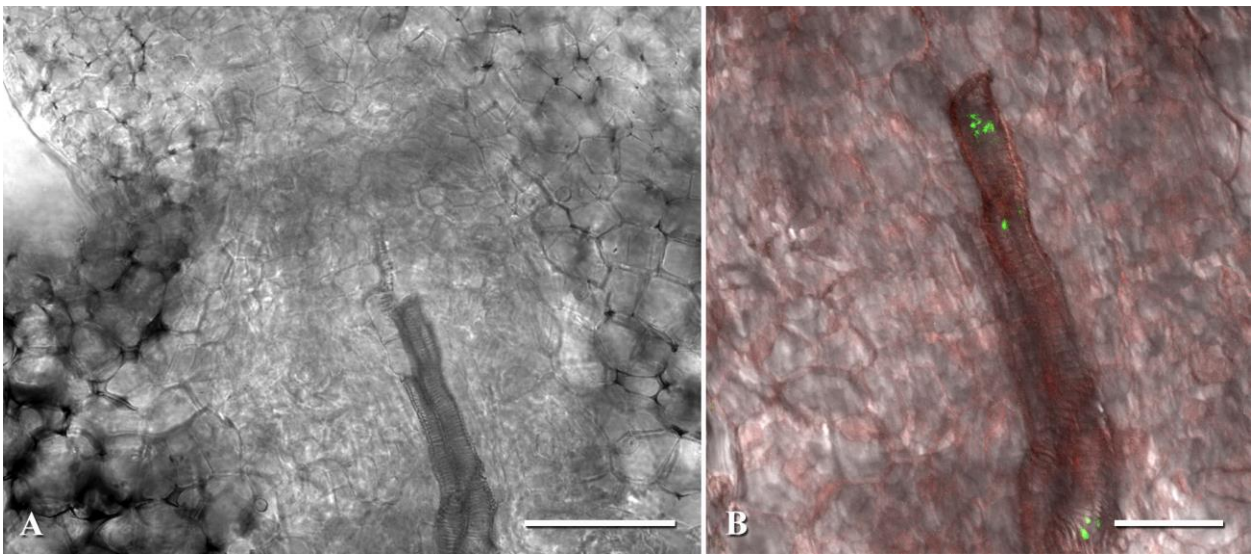

Figure 2.6: Systemic inoculation of peduncle truss resulting in the colonization of xylem vessels within the funiculus of a developing tomato seed. (A) Differential interference contrast (DIC) image of xylem vessels leading into the developing seed. (B) Magnification of panel A showing xylem with eGFP-labeled *Clavibacter michiganensis* subsp. *michiganensis* colonization. Confocal image was acquired by merging 3 channels (488 nm, 543 nm, and transmitted light). Scale bars: 50 µm in A; 20 µm in B.

Pedicel systemic inoculation. In addition to peduncle truss inoculations, the pedicels of individual floral units were also inoculated (Figure 2.2) to more closely follow fruit infection by *C. michiganensis* subsp. *michiganensis*. Fruit harvested 14 DPI appeared visually healthy, but extensive xylem colonization was observed (Table 2.5). Unlike peduncle inoculations, pedicel inoculations caused discoloration and premature abscission of the remaining vine-attached fruit within 4-5 weeks. Of the five green fruit that could be studied, one had been inoculated at the flower stage, and two each at the emerging fruit and >1 cm fruit stages. Large numbers of *C. michiganensis* subsp. *michiganensis* could be seen throughout the pericarp xylem of all inoculated fruit, and at high numbers on multiple seed of one fruit (Figure 2.7). It appeared that *C. michiganensis* subsp. *michiganensis* colonized both the intra-and intercellular regions of the

developing seed. Negative control fruit had no discoloration or colonization by *C. michiganensis* subsp. *michiganensis.*

Table 2.5: *Clavibacter michiganensis* subsp. *michiganensis* movement and colonization of tomato fruit tissue via systemic inoculation at the pedicel

Inoculation stage[a]	No. of fruit	No. of infected fruit	DPI[b]	No. of fruit with Cmm colonization		
				Xylem	Base of fruit[c]	Seed[d]
Flower	1	1	14	1	1	1
Emerging fruit	2	2	14	2	1	0
≥1 cm fruit	2	2	14	2	0	0
Total	5	5	-	5	2	1

[a] Growth stage at which the pedicels were inoculated.
[b] Days post inoculation (DPI) when harvested.
[c] Vascular bundles colonized by *Cmm* at the base of the fruit represent the maximum distance bacteria traveled from the calyx.
[d] The number of fruit with visible seed colonization are shown.

DISCUSSION

Stable transformation of *C. michiganensis* subsp. *michiganensis* with constitutive eGFP-expression permitted detailed *in situ* visualization of the entry routes used to colonize tomato fruit and seed. Transformation efficiency was not equal among the four field strains, but differences in exopolysaccharide composition and electrocompetence may be partially responsible (Yoshida et al. 2001). Among the ten eGFP-isolates studied, differences in fluorescence and virulence were observed; yet, all reached similar *in planta* populations at one centimeter above the inoculation point.

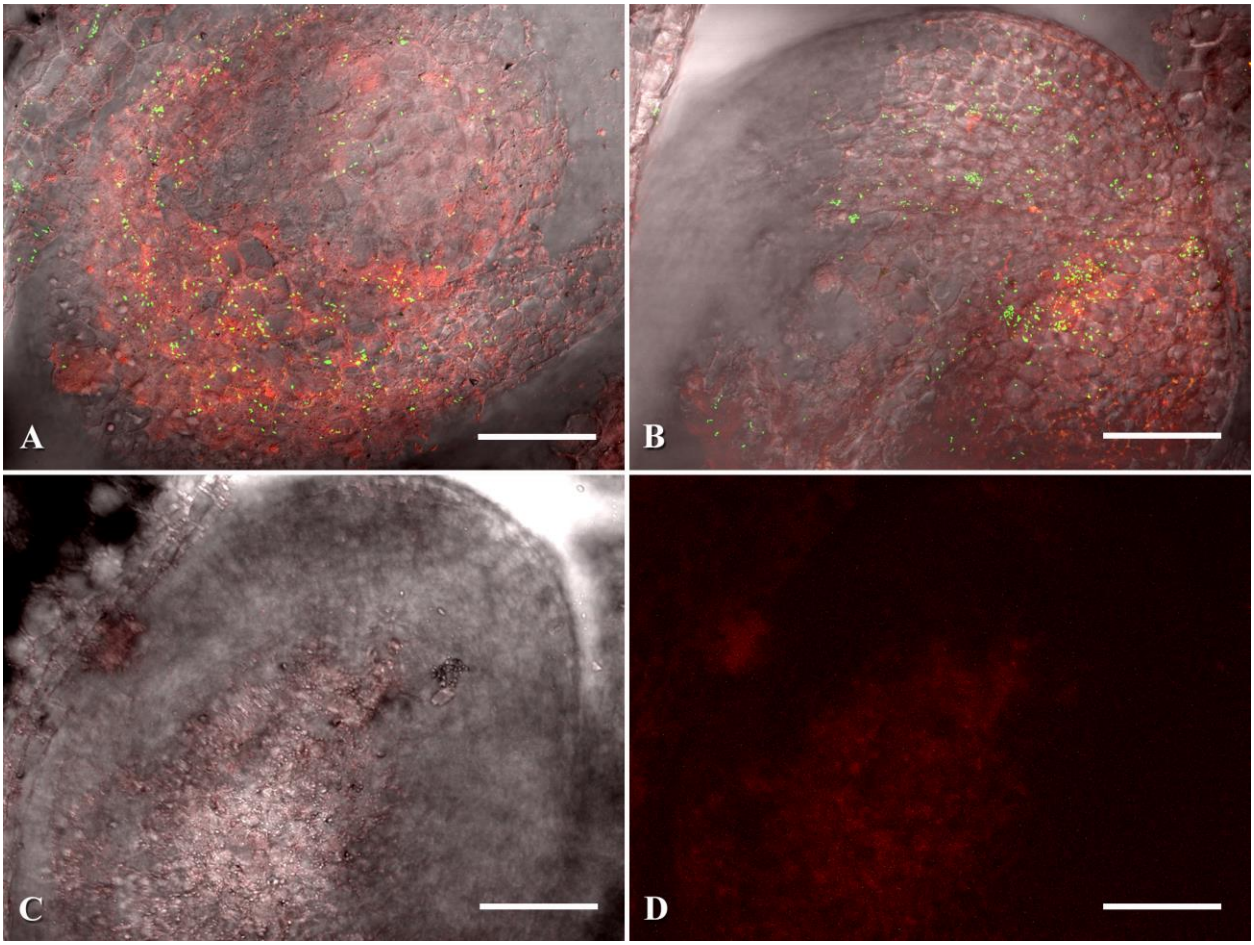

Figure 2.7: Systemic inoculation of pedicel with subsequent tomato seed colonization. (A-B) eGFP-labeled *Clavibacter michiganensis* subsp. *michiganensis* colonization of developing seeds. (C) Negative control seed at same developmental stage. (D) Same image as panel C, but only 2 channels are shown (488 nm and 543 nm). Microscopy images (A-C) were generated by merging 3 channels (488 nm, 543 nm, and transmitted light). Scale bars: 50 µm.

Additionally, two isolates were numerically (although not statistically) more aggressive than their wild-type field strain. The reason for these differences is unknown, but may due to the site of transposon integration.

Bird's-eye lesions were originally believed to be superficial blemishes, in a similar manner to bacterial speck or spot of tomato (Bryan 1930); however, eGFP-labeled *C. michiganensis* subsp. *michiganensis* actively grew within lesions on the exocarp of tomatoes and subsequently spread into the fruit when externally inoculated. Confinement of bacteria to the

inter- and intracellular regions of the lesions during the earlier stages of fruit development was quickly lost once fruit began to ripen (breaker stage), suggesting that the weakened cellular components facilitated bacterial spread into the thin-walled mesocarp cells and xylem of the pericarp. Once in the xylem, the pathogen spread through the fruit and entered the plant through the pedicel. *C. michiganensis* subsp. *michiganensis* is known to possess an arsenal of extracellular cell-wall-degrading-enzymes, which could become more effective in combination with natural fruit softening processes (Gross and Wallner 1979; Eichenlaub and Gartemann 2011; Gartemann et al. 2008). In one case, an externally inoculated fruit had mottling symptoms on an individual locular cavity, which appeared as a subepidermal "lesion" and was beginning to liquefy beneath the intact epidermis. This mottling symptom had been previously observed only in systematically infected greenhouse tomato fruit (Layne and Rainforth 1966). These observations suggest that the external inoculation of a tomato fruit with *C. michiganensis* subsp. *michiganensis* can yield both (i) external bird's-eye lesions that ingress into the fruit pericarp and eventually to the seed, and (ii) systemically produced subepidermal "lesions" that egress from the inner fruit vasculature, in a similar manner to *Xanthomonas campestris* pv. *pruni* (Du Plessis 1990; Layne and Rainforth 1966).

The exact method of entry into the fruit from external sources is still unclear. Tomato fruit do not possess any stomata and wounds are not necessary for lesion development (15). Therefore, the pathogen is thought to access the fruit via glandular and nonglandular trichomes that cover developing green fruit. As the fruit begins to grow it sheds trichomes, which exposes open trichome bases and provides points of entry, as has been shown for *Pseudomonas syringae* pv. *tomato* (Getz et al. 1983; Bryan 1930). To our knowledge, the brush inoculation method does not produce any wounds (Medina-Mora et al. 2001), but unidentified wounds could have

been present on the fruit exocarp. However, the consistency of fruit xylem colonization, in addition to the movement of *C. michiganensis* subsp. *michiganensis* into the fruit mesocarp suggests active pericarp infiltration. Lesion-to-vascular ingression most likely occurred near the distal end of the fruit where vascular elements were noticeably closer to the epidermis. Fruit pericarp penetration has also been observed with *Xanthomonas campestris* pv. *pruni* (bacterial spot of *Prunus*) and *Pseudomonas syringae* pv. *lachrymans* (angular leaf spot of cucurbits), which actively penetrate several millimeters into fruit mesocarp (Du Plessis 1990; Wiles and Walker 1951).

The systemic invasion of tomato fruit by *C. michiganensis* subsp. *michiganensis* has been previously observed (Layne and Rainforth 1966; Bryan 1930). However, no detailed histopathological study had been performed on the routes of seed infection (Singh and Mathur 2004). To see the impact of different stages of fruit development on fruit and seed infection by *C. michiganensis* subsp. *michiganensis*, floral units were systemically inoculated at various stages of growth. The highest fruit infection rate, via systemic inoculation, occurred during the dead flower/emerging fruit stage with a 78% infection rate; however, seed infections were more common when the floral units were between the bud and pre-anthesis flower stages. In peduncle truss inoculations, seed internalization was not directly visualized, but *C. michiganensis* subsp. *michiganensis* could be observed within the funiculus. Similarly, *Xanthomonas campestris* pv. *campestris* was rarely seen within the seed of systemically-inoculated cabbage, with most cases being confined to the funiculus, which dries and adheres to the seed coat (Cook et al. 1952). Conversely, pedicel inoculations led to intra-and intercellular colonization of the developing seed. The ability of *C. michiganensis* subsp. *michiganensis* to access the xylem, funiculus, and seed, in combination with no external fruit or plant symptoms (as in the peduncle truss

inoculations) highlights the difficulty in identifying diseased plants with potentially contaminated seed.

The intracellular colonization of pericarp cells was consistently observed in fruit from both external and systemic inoculations. Intracellular colonization by phytobacteria is rarely observed, but it has been documented with *Pseudomonas syringae* pv. *lachrymans* and *Xanthomonas campestris* pv. *cajani* during seed infections (Wiles and Walker 1951; Sharma et al. 2001), as well as with Gram-positive phytobacteria like *Streptomyces ipomoes* and *S. turgidiscabies* (Joshi et al. 2007; Clark and Matthews 1987; Hogenhout and Loria 2008). In previous studies, *C. michiganensis* subsp. *michiganensis* had been primarily observed within the intercellular spaces of tomato leaves and stems, with intracellular populations occurring later in disease development (Carlton et al. 1998; Bryan 1930; Wallis 1977), yet intact fruit cells appeared to be colonized in this study (Figure 3).

Irrespective of inoculation method (systemic or external), *C. michiganensis* subsp. *michiganensis* was observed within and around multiple developing tomato seed. *C. michiganensis* subsp. *michiganensis* cells were located within the developing seed, endosperm, and funiculus, although at relatively low levels compared with the large numbers of cells observed in xylem and pericarp cells. These results highlight the difficulty in detecting and eradicating small initial pathogen populations within seed lots. Yet, as populations reach exponential growth either during transplant production or in the field, rapid spread will occur and visual symptoms will appear (Werner et al. 2002). The data presented here demonstrate that *C. michiganensis* subsp. *michiganensis* can actively infect tomato seeds both systemically through the xylem and externally via fruit lesions. But, complete comprehension of fruit pathogenesis by *C. michiganensis* subsp. *michiganensis* is still in its infancy since avirulent strains (negative in

hypersensitive response assays) have been shown to produce external fruit lesions (Medina-Mora et al. 2001), and *C. michiganensis subsp. michiganensis* appears to be actively moving within the fruit pericarp, even though its purported to be non-motile (Medina-Mora et al. 2001; Bryan 1930). These and other questions will require further experimentation.

ACKNOWLEDGEMENTS

This research was supported by grant no. IS-4403-11C from the United States-Israel Binational Agricultural Research and Development (BARD) Fund. Support for M. Tancos was additionally provided by a fellowship from Cornell University College of Agriculture and Life Sciences and the National Science Foundation-Graduate Research Fellowships Program (GRFP) grant no. DGE-1144153. The authors thank Kent Loeffler for image organization and Lisa Jones, Holly Lange, and Amara Dunn for technical assistance.

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CHAPTER 3

PLANT-LIKE BACTERIAL EXPANSINS PLAY CONTRASTING ROLES IN TWO TOMATO VASCULAR PATHOGENS

ABSTRACT

Expansins, which loosen plant cell walls, play critical roles in normal plant growth and development. The horizontal acquisition of functional plant-like expansin genes in numerous xylem-colonizing phytopathogenic bacteria suggests the importance of bacterial expansins in plant colonization. To investigate the role of bacterial expansins in plant diseases, we mutated the non-chimeric expansin genes of two xylem-inhabiting bacterial pathogens, the Gram-positive bacterium *Clavibacter michiganensis* subsp. *michiganensis* (*Cmm*) and the β-proteobacterium *Ralstonia solanacearum* (*Rs*). The *Cmm CmEXLX2* mutant had increased symptom development on tomato, which was characterized by greater vascular necrosis and the proliferation of atypical lesions on distant petioles. This increased disease severity correlated with larger mutant *in planta* populations even though no differences in growth were observed between strains *in vitro*. Similarly, when inoculated onto fruit, the *CmEXLX2* mutant produced significantly larger lesions with larger necrotic centers. In contrast, the *Rs RsEXLX* mutant had reduced virulence on tomato following root inoculation, but not following a direct petiole inoculation, suggesting that the RsEXLX expansin contributes to early root colonization. Consistent with this finding, the *RsEXLX* mutant had increased attachment to seedling roots, which may hinder the bacteria from

entering the plants vasculature. Overall, these results demonstrate the diverse roles of non-chimeric bacterial expansins and their importance in plant-bacterial interactions.

INTRODUCTION

Plant primary cell walls are highly dynamic structures that fluctuate between a rigid or relaxed state, enabling basic biological processes such as growth, enlargement, and division (Cosgrove 2005, 1993). Modulating elasticity and plasticity is an intricate process that requires many cell wall loosening enzymes, notably expansins (Cosgrove 1993; Sampedro and Cosgrove 2005). Plant expansins loosen the rigid carbohydrate matrix of the cell wall through an uncharacterized non-lytic slippage mechanism (Cosgrove 2000). This key function has led to the conservation of expansins in all land plants, with isoforms regulating cell wall changes associated with cellular growth, vascular differentiation, fruit ripening, seed germination, abscission, and leaf development (Kende et al. 2004; Im et al. 2000; Rose et al. 1997; Cho and Cosgrove 2000). Expansins are pH-dependent and become active when plant growth hormones stimulate H^+-ATPases, which produce a proton differential across the plasma membrane (Cosgrove 2000).

Recent evidence suggests microbes independently acquired and exploited this plant-derived enzyme for unknown reasons. Nikolaidis et al. (2014) suggested multiple independent horizontal gene transfers (HGT) have led to the microbial acquisition of plant expansins with subsequent HGT events within bacterial and fungal phyla (Nikolaidis et al. 2014). Only 3% of sequenced bacteria acquired and maintained these expansin xenologs, but they include a diverse range of bacterial genera from free-living saprophytes to plant pathogens (Nikolaidis et al. 2014). Interestingly, of the plant pathogenic bacteria that possess an expansin gene, all genera (except

Streptomyces) contain members that are systemic xylem pathogens including *Xanthomonas*, *Xylella*, *Ralstonia*, *Dickeya*, *Pectobacterium*, *Acidovorax*, and *Clavibacter* (Nikolaidis et al. 2014; Georgelis et al. 2015). However, the expansin xenolog is not present in the non-vascular phytopathogenic bacterial genera such as *Pseudomonas* or *Agrobacterium* (Nikolaidis et al. 2014). Bacteria are known to have two forms of expansins: a chimeric version in which the expansin is fused to an endoglucanase and a non-chimeric version (Nikolaidis et al. 2014; Jahr et al. 2000). For the majority of bacteria, only a single expansin (either chimeric or non-chimeric) is present with the exception of the tomato pathogen *Clavibacter michiganensis* subsp. *michiganensis* (*Cmm*), which possess both a chimeric (*CmEXLX1*) and non-chimeric version (*CmEXLX2*) (Nikolaidis et al. 2014; Georgelis et al. 2015). In contrast, another vascular tomato pathogen, *Ralstonia solanacearum* (*Rs*) has only a non-chimeric expansin (Nikolaidis et al. 2014).

Cmm, the internationally quarantined causal agent of bacterial canker in tomato (*Solanum lycopersicum*), is an economically devastating seed-disseminated phytopathogen that is present throughout major tomato-producing regions (Bryan 1930; de León et al. 2011). *Cmm* enters the plant epiphytically through natural openings, wounds or infected seed (Bryan 1930; Tancos et al. 2013; Carlton et al. 1998). Once inside a plant this Gram-positive bacterium systemically spreads throughout the vasculature, plugging and degrading xylem vessels, resulting in tissue maceration and the impairment of water transport, leading to characteristic wilting, marginal necrosis of leaflets, stem cankers, and fruit lesions (Wallis 1977; Tancos et al. 2013; Bryan 1930). Bacterial canker of tomato is difficult to control due to the lack of resistant cultivars and improper sanitation (Werner et al. 2002; Sen et al. 2013).

In contrast to *Cmm*, *Rs* is a soil-borne Gram-negative vascular pathogen that infects a wide host range of economically important crops, including monocots and dicots (Salanoubat et al. 2002). *Rs* infects roots near the elongation zone, at sites of secondary root emergence, and through wounds (Vasse et al. 1995). Once the root cortex is infected, *Rs* penetrates the vascular cylinder and colonizes the xylem. The pathogen spreads systemically throughout the vasculature, reaching population sizes $>10^9$cfu/gm of stem tissue. Wilting symptoms result when xylem vessels become occluded by the mass of bacterial cells and extracellular polymeric substances (Vasse et al. 1995). Like *Cmm*, *Rs* is difficult to manage due to the lack of resistant plant cultivars or other practical control strategies (Bae et al. 2015).

Microbial expansins share high structural similarity to their plant expansin xenologs, and unique binding domains are highly conserved (Kerff et al. 2008; Pastor et al. 2015). Purified bacterial expansins from *Bacillus subtilis, Xanthomonas campestris, Rs*, and *Cmm* have all been shown to loosen plant cell walls *in vitro* without lytic activity, but effects appear to be modest relative to plant expansins (Georgelis et al. 2014; Kerff et al. 2008; Bunterngsook et al. 2015). The function of non-chimeric bacterial expansins remain unknown, especially in phytopathogenic species, although the disruption of the non-chimeric expansin gene in the saprophyte biocontrol agent *B. subtilis* led to a significant reduction in attachment to maize roots (Kerff et al. 2008). In contrast, disrupting the chimeric expansins (expansin fused to an endoglucanase) from the phytopathogenic bacteria *Xylella fastidiosa* and *Cmm* reduced disease symptoms (Ingel et al. 2015; Jahr et al. 2000).

Here we explore the influence of non-chimeric bacterial expansins on pathogenesis and colonization of tomato. Initial studies were performed with the Gram-positive bacterium *Cmm*, but to further explore the function of non-chimeric bacterial expansins we included the Gram-

negative bacterium *Rs.* Our working hypothesis was that non-chimeric expansins from *Cmm* and *Rs* would contribute to vascular colonization. The objectives of the study were to explore the role of the non-chimeric *Cmm* expansin CmEXLX2 in (i) disease progression and symptom development in tomato, (ii) vascular colonization and systemic movement, and (iii) compare the roles of non-chimeric expansins for the two tomato vascular pathogens *Cmm* and *Rs*.

RESULTS

We took a genetic approach to investigate the role of non-chimeric plant-like bacterial expansins in the pathogenesis of two vascular plant pathogenic bacteria. A virulent *Cmm* New York field strain was selected to disrupt *CmEXLX2* since it naturally lacked the plasmid-borne chimeric version *CmEXLX1* (Tancos et al. 2015). To create the Δ*CmEXLX2* expansin mutant, the native *CmEXLX2* gene of *Cmm* strain Cmm0317 was replaced with a *CmEXLX2*::Gm^r cassette without downstream frameshift mutations. The CΔ*CmEXLX2*$^+$ complement was created by introducing the expansin gene and its native promoter onto a low-copy number plasmid. As expected, the mutant did not express *CmEXLX2* while the wildtype and complement expressed *CmEXLX2* (Figure 3.1). The *Rs* expansin mutant *RsEXLX* was created by unmarked deletion of the *RsEXLX*

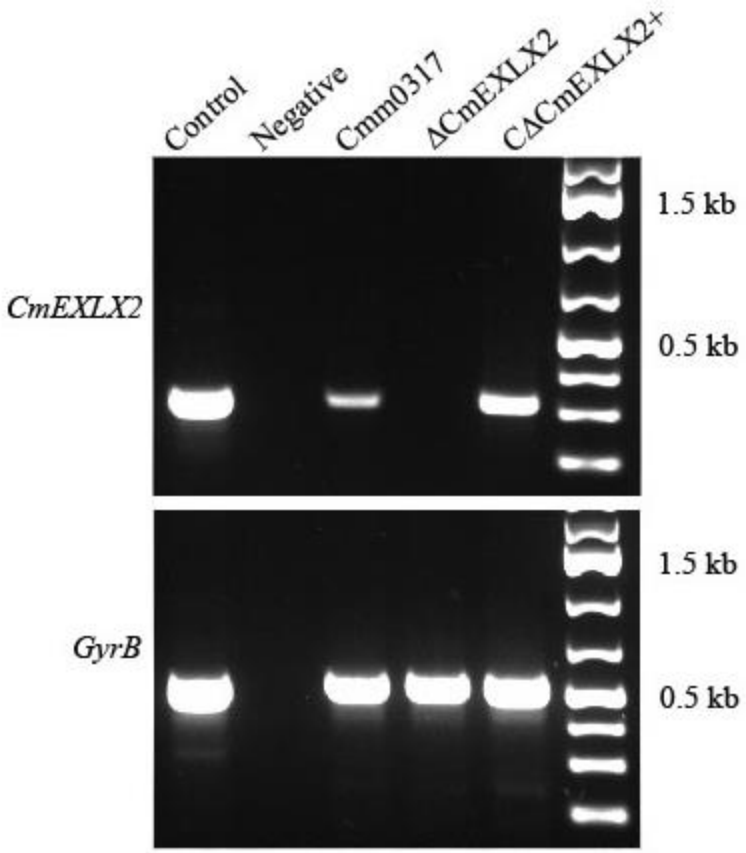

Figure 3.1. Analysis of expression of *CmEXLX2 in vitro*. RT-PCR of *CmEXLX2* with RNA obtained from wild-type (Cmm0317), *CmEXLX2* mutant (Δ*CmEXLX2*), and complement (CΔ*CmEXLX2*$^{+}$) strains (top gel). As a constitutive control, housekeeping gene *GyrB* was amplified using the same RT-PCR conditions (bottom gel).

gene using *sacB* positive selection. The *in vitro* growth curves of the *CmEXLX2* mutant were indistinguishable from those of wildtype Cmm0317 in both nutrient-rich LB medium (*P*=0.102) and nutrient-poor tomato xylem sap (*P*=0.119) demonstrating that *CmEXLX2* did not influence *Cmm* growth (Figure 3.2). Growth was not influenced in the *Rs* expansin mutant, with similar growth to wildtype in both rich (*P*=0.493) and minimal medium (*P*=0.301) (Figure 3.3). Additionally, the mutation in *CmEXLX2* did not influence host recognition in *Mirabilis jalapa* (four o'clock plants), where Δ*CmEXLX2* induced a strong hypersensitive response (HR) similar to both the wildtype and complemented mutant (Table 3.1).

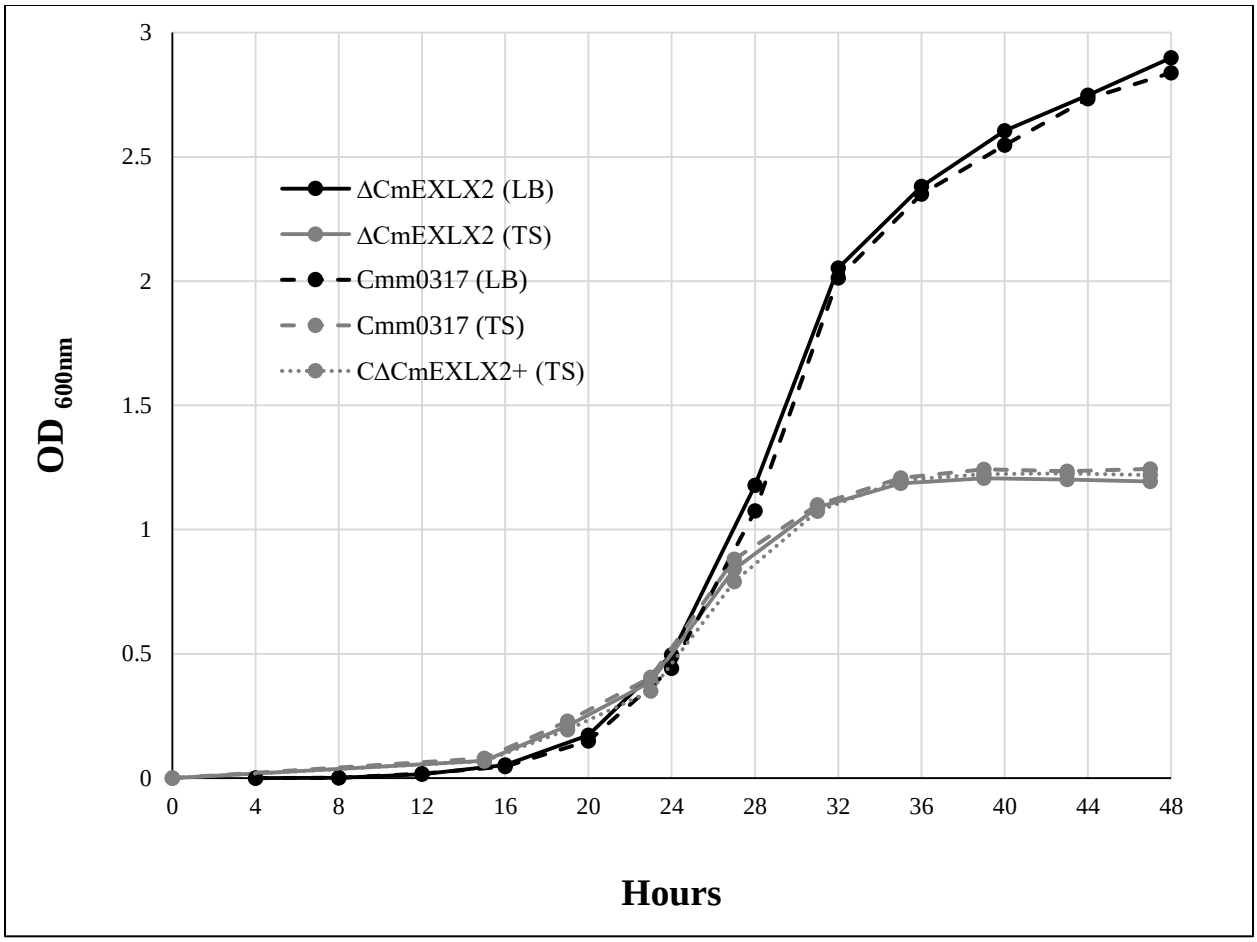

Figure 3.2. Growth of *Clavibacter michiganensis* subsp. *michiganensis* Cmm0317 (wild-type), Δ*CmEXLX2* (expansin mutant), and CΔ*CmEXLX2*$^{+}$ (complement) in nutrient-rich LB medium (LB = black lines) and in nutrient-poor tomato xylem sap medium (TS = gray lines).

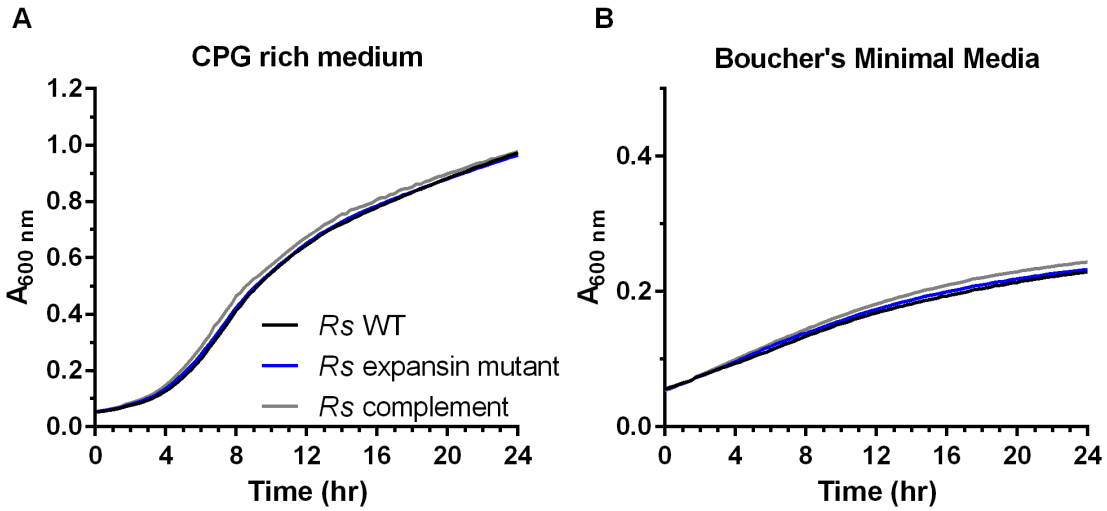

Figure 3.3. *In vitro* growth of *Ralstonia solanacearum* strains. Growth of *R. solanacearum* WT (black lines), *R. solanacearum* expansin mutant (blue lines), and *R. solanacearum* complement (grey lines) in (A) CPG rich medium or (B) Boucher's minimal media.

Table 3.1. Virulence of *Clavibacter michiganensis* subsp. *michiganensis* strains on tomato plants

Strain	HR[†]	AUDPC[‡]	Disease incidence[§]	Petiole lesion length (cm)[¶]	CFU/g in *planta*[#]	
					9 DPI	21 DPI
Cmm0317	+	139 (± 36) ^{B}	15/15	0	2.78×10^{8} ^{B}	5.36×10^{8} ^{C}
Δ*CmEXLX2*	+	430 (± 64) ^{A}	15/15	2.16 (±0.70)	3.72×10^{9} ^{A}	4.82×10^{9} ^{A}
CΔ*CmEXLX2*$^{+}$	+	157 (± 21) ^{B}	15/15	0.59 (±0.21)	2.25×10^{8} ^{B}	2.30×10^{9} ^{B}
Water	-	0.0	0/15	0	0.0	0.0

[†]Induction of a hypersensitive response (HR) in *Mirabilis jalapa.* (+) positive for HR reaction; (-) negative for HR reaction.

[‡]The mean area under the disease progress curve (AUDPC) for disease severity for three independent experiments is given. Significant differences among strains were tested with PROC GLIMMIX ($P<0.05$) followed by Tukey-Kramer posttest ($P<0.05$). AUDPC values followed by the same superscript are not significantly different. Numbers in parentheses indicate the standard error associated with the AUDPC values.

[§]Expressed as the number of wilting plants/number of inoculated plants characterized at 21 days post inoculation.

[¶]The mean length of lesions located on the inoculated petiole at 21 days post inoculation. Numbers in parentheses indicate the standard error associated with the lesion lengths.

[#]The mean *in planta* population recovered from a 0.5 cm section of tomato stem tissue located 1-cm above the inoculation site at both 9 and 21 days post inoculation. Significant differences among strains were tested by PROC GLIMMIX ($P<0.05$) followed by Tukey-Kramer posttest ($P<0.05$). *In planta* populations followed by the same superscript are not significantly different.

Plant expansins facilitate cell wall loosening as plant cells modify and grow. To test the hypothesis that non-chimeric bacterial expansins influence peptidoglycan loosening in *Cmm* and *Rs*, an autolysis assay was performed, which measures the susceptibility of bacterial cell walls to lysing. Autolysis of the *Cmm* and *Rs* expansin mutants did not differ from their wildtype and complement strains (P=0.141 for *Cmm* and P=0.086 for *Rs*) (Figure 3.4).

The *Cmm* Δ*CmEXLX2* mutant causes increased symptom development on tomato seedlings and fruit. Tomato seedlings infected with the *Cmm* Δ*CmEXLX2* mutant consistently developed wilt symptoms earlier and with increased severity compared to the wildtype and complemented mutant (P<0.0001) (Table 3.1). Sixty-percent of the plants began to wilt as early as 10 days post inoculation (DPI) when infected with Δ*CmEXLX2*, 15 DPI with wildtype, and 13 DPI with the complemented mutant. All three *Cmm* strains produced typical stem cankers at the site of inoculation, but the *Cmm* expansin mutant also produced large cankerous lesions within the petiole vascular bundles of distant leaves (Table 3.1, Figure 3.5). These atypical petiole lesions extended along the tracks of the vascular bundles with necrosis being confined to the vasculature and healthy tissue surrounding the lesions, until later stages of disease (Figure 3.5). Petiole lesions appeared approximately 11-14 DPI and were present on multiple leaves of the tomato seedling. These lesions were never observed on plants inoculated with the wildtype strain, and only rarely present on plants inoculated with the complemented mutant.

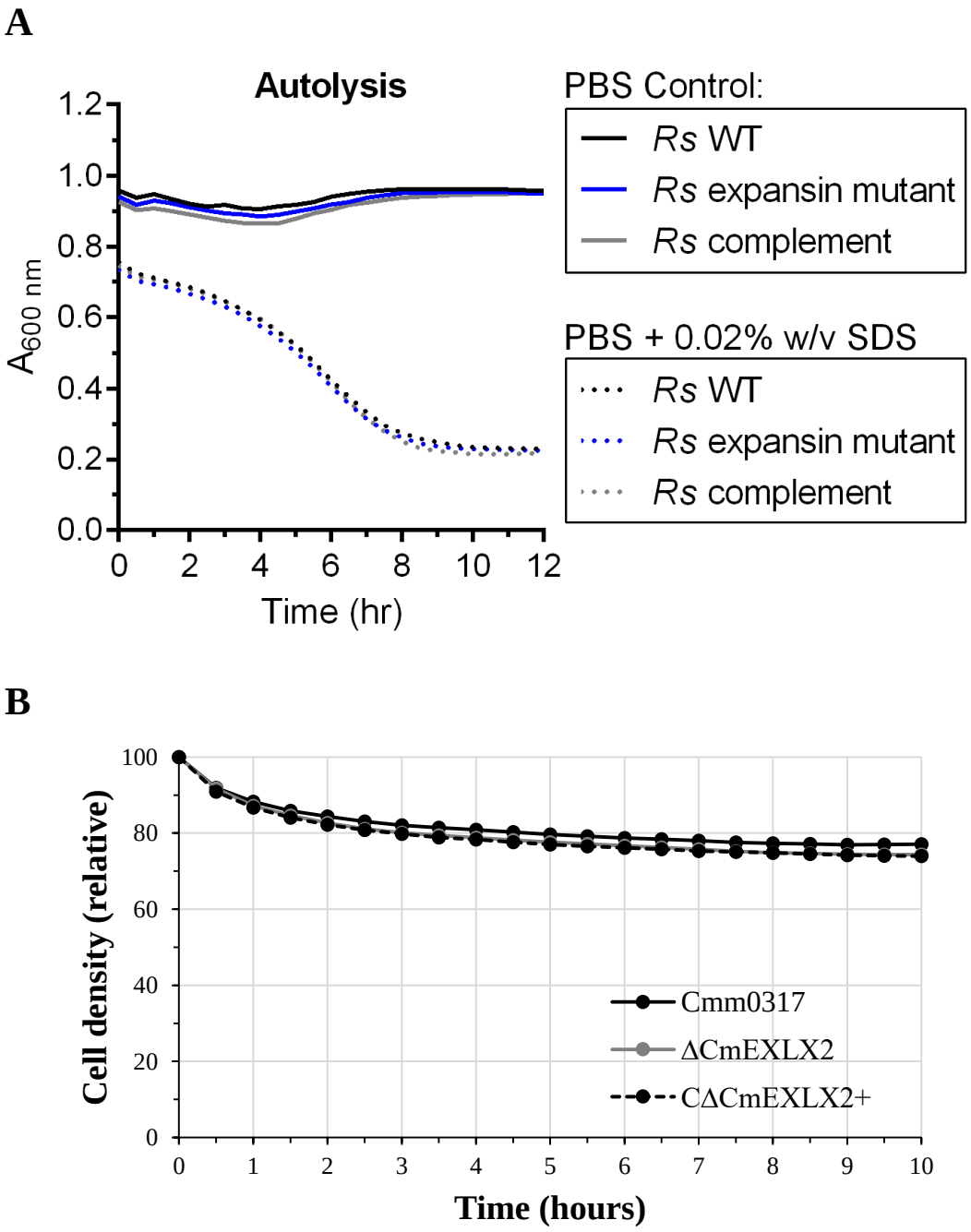

Figure 3.4. Autolysis of *Ralstonia solanacearum* and *Clavibacter michiganensis* subsp. *michiganensis* strains. (A) *R. solanacearum* strains were incubated in PBS or in PBS with 0.02% w/v SDS. (B) *C. michiganensis* subsp. *michiganensis* strains incubated in PBS with 0.02% w/v SDS.

In planta bacterial populations were significantly different at 1 cm above the inoculation site at both 9 DPI (P=0.0048) and 21 DPI (P<0.0001) (Table 3.1). To determine if systemic movement influenced symptom development and *in planta* population sizes, *Cmm* populations were also assessed 5 and 10 cm above the inoculation site at 21 DPI. All three *Cmm* strains were present 5 and 10 cm above the site of inoculation, suggesting there were no differences in acroptetal movement within the stem xylem (Table 3.2). Consistent with our findings at 1 cm above the inoculation site, the Δ*CmEXLX2* population at 5 cm was significantly larger (P=0.024) than wildtype with populations of $2.41x10^9$ and $6.85x10^8$ CFU/gram of tissue, respectively. However, at 10 cm above the inoculation site, there were no significant differences between bacterial populations (P=0.46) (Table 3.2).

Figure 3.5. Atypical tomato petiole lesions associated with the *Clavibacter michiganensis* subsp. *michiganensis* expansin mutant Δ*CmEXLX2*. (A) An individual petiole lesion rupturing a vascular bundle. Necrosis appeared confined to the vasculature with healthy plant tissue surrounding the petiole lesions. (B) Multiple lesions localized to the vasculature of an individual petiole. Black arrows highlight the large cankerous lesions present along multiple vascular tracks.

The plant vasculature regulates the transportation of water and nutrients with a network of vascular bundles comprised of aggregated xylem vessels and phloem tissue. To determine if the CmEXLX2 expansin influences movement of *Cmm* between xylem vessels or vascular bundles, we inoculated EGFP-expressing *Cmm* strains into tomato plants and visualized intra- or intervascular colonization. EGFP-expressing *Cmm* wildtype (*CmEXLX2*$^{+}$) and Δ*CmEXLX2* strains colonized xylem vessels 3 cm above and below the inoculation site at 5, 7, and 9 DPI (Table 3.3A-B, Figure 3.6). No differences were observed in xylem colonization, lateral movement or parenchyma cell colonization between the strains at any time point (5, 7, or 9 DPI) or distance (3 cm below to 3 cm above the inoculation site). By 9 DPI, *Cmm* strains were present at high concentrations resulting in the maceration of vessel elements and the colonization of surrounding parenchyma cells (Table 3.3B).

Table 3.2. *In planta* populations of *Clavibacter michiganensis* subsp. *michiganensis* strains colonizing tomato stems 5 and 10-cm above the site of inoculation following 21 days post inoculation.

Strain	CFU/g i*n planta*[†]	
	5-cm above inoculation site	10-cm above inoculation site
Cmm0317	6.85×10^{8} B	1.52×10^{9} A
Δ*CmEXLX2*	2.41×10^{9} A	2.06×10^{9} A
CΔ*CmEXLX2*$^{+}$	8.29×10^{8} AB	9.75×10^{9} A
Water	0.0	0.0

[†]The mean *in planta* population recovered from a 0.5 cm section of tomato stem tissue located above the inoculation site at 21 days post inoculation. Significant differences among strains were tested by ANOVA ($P<0.05$) followed by Tukey's studentized range posttests ($P<0.05$). *In planta* populations followed by the same superscript are not significantly different.

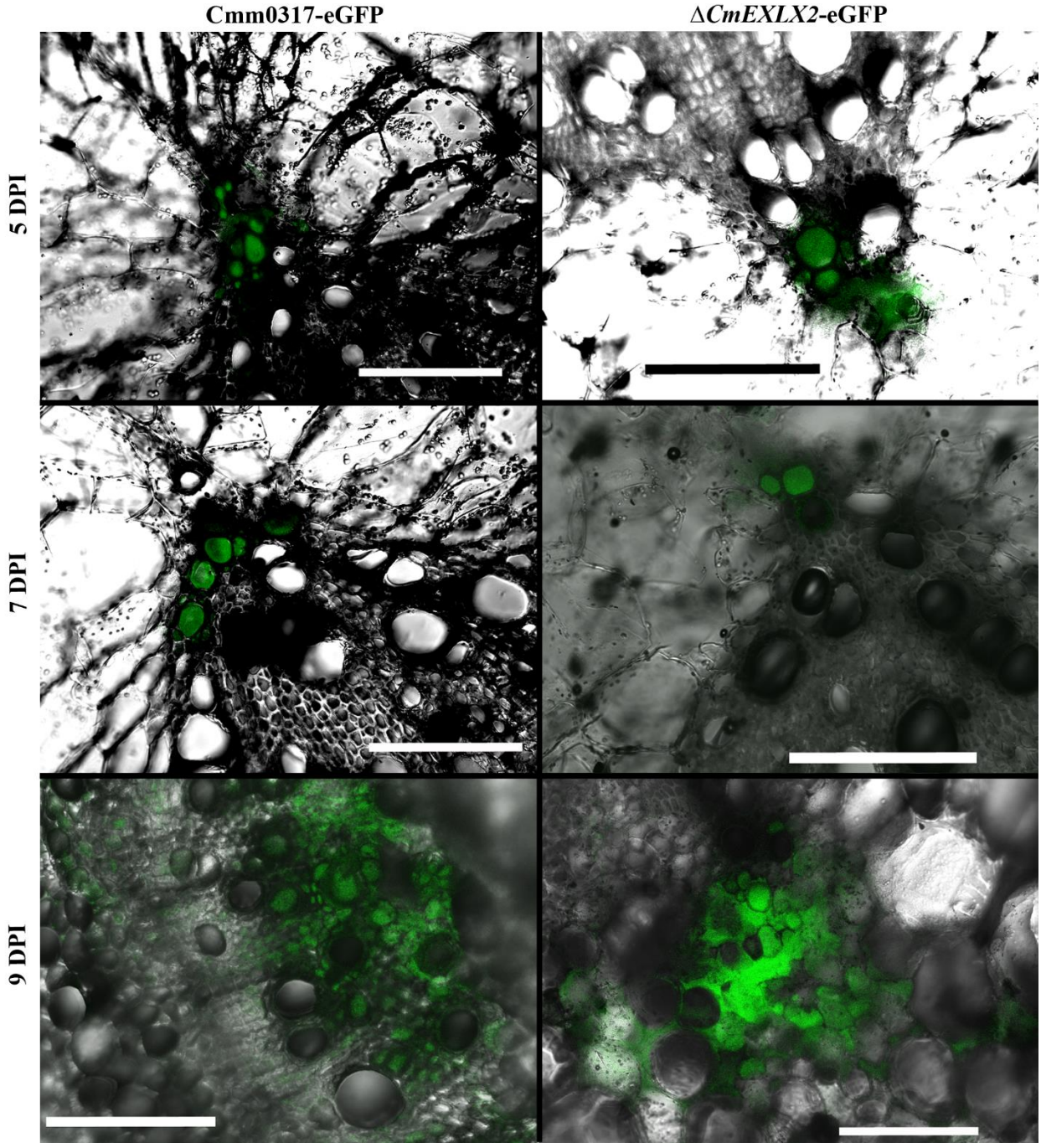

Figure 3.6. Inter-and intravascular colonization of tomato xylem vessels infected with eGFP-*Clavibacter michiganensis* subsp. *michiganensis* Cmm0317 (wild-type) and Δ*CmEXLX2* (expansin mutant) at specified days post inoculation (DPI). All confocal microscopy images were generated by merging 2 channels (488 nm and transmitted light). Scale bar: 200 µm.

Table 3.3. *Clavibacter michiganensis* subsp. *michiganensis* colonization and vasculature movement within tomato stem tissue with eGFP- expressing wild-type and *CmEXLX2* mutant strains. A) Amount of colonization and movement present at 5 and 7 days post inoculation. B) Amount of colonization and movement present at 9 days post inoculation.

A.

Days post inoculation	Distance from inoculation	No. of infected protoxylem[†]		Percent of infected vascular bundles[‡]	
		Cmm0317	Δ*CmEXLX2*	Cmm0317	Δ*CmEXLX2*
5 DPI	3 cm	4.2	4.3	24.8%	21.2%
	2 cm	3.7	2.7	27.8%	18.0%
	1 cm	2.8	3.3	25.6%	18.8%
	-1 cm	6.5	11.3	50.8%	77.5%
	-2 cm	4.2	6.0	50.0%	51.7%
	-3 cm	2.7	2.3	28.3%	28.3%
7 DPI	3 cm	9.8	4.2	27.0%	21.7%
	2 cm	7.0	8.2	35.1%	31.5%
	1 cm	8.2	8.8	48.9%	38.5%
	-1 cm	8.7	13.5	48.7%	60.0%
	-2 cm	5.0	4.3	54.2%	54.2%
	-3 cm	3.5	4.2	39.2%	45.8%

B.

Days post inoculation	Distance from inoculation	No. of infected vascular bundles (multi-infection)[§]		No. of infected vascular bundles (singularly- infected)[¶]		Percent of infected vascular bundles[‡]	
		Cmm0317	Δ*CmEXLX2*	Cmm0317	Δ*CmEXLX2*	Cmm0317	Δ*CmEXLX2*
9 DPI	3 cm	4.2	2.7	2.2	1.7	55.5%	48.4%
	2 cm	5.4	4.2	2.0	2.0	67.4%	64.3%
	1 cm	4.6	3.7	2.2	1.2	67.5%	64.5%
	-1 cm	3.0	1.5	1.0	1.7	100.0%	79.2%
	-2 cm	1.0	1.3	1.4	1.8	55.0%	79.2%
	-3 cm	0.2	0.8	2.4	2.0	65.0%	66.7%

[†]Mean number of infected protoxylem vessels present at each distance interval.
[‡]Mean percentage of vascular bundles infected at each respective distance interval.
[§]Mean number of infected vascular bundles with *C. michiganensis* subsp. *michiganensis* present in vessels and parenchyma cells at high concentrations. Infected vascular bundles were too heavily colonized to determine the exact number of infected vessel elements.
[¶]Mean number of infected vascular bundles with *C. michiganensis* subsp. *michiganensis* present at low enough concentrations that individual infected vessels could be identified.

When inoculated onto immature green fruit, all three *Cmm* strains produced typical lesions. Fruit lesions appeared 3-4 DPI, but phenotypic differences between strains became evident 7-10 DPI. The *Cmm* Δ*CmEXLX2* mutant lesions were significantly larger and more necrotic than both the wildtype and complemented mutant fruit lesions (P<0.0001), but they had a significantly thinner 'white halo' compared to the wildtype lesions (P=0.0008) (Figure 3.7).

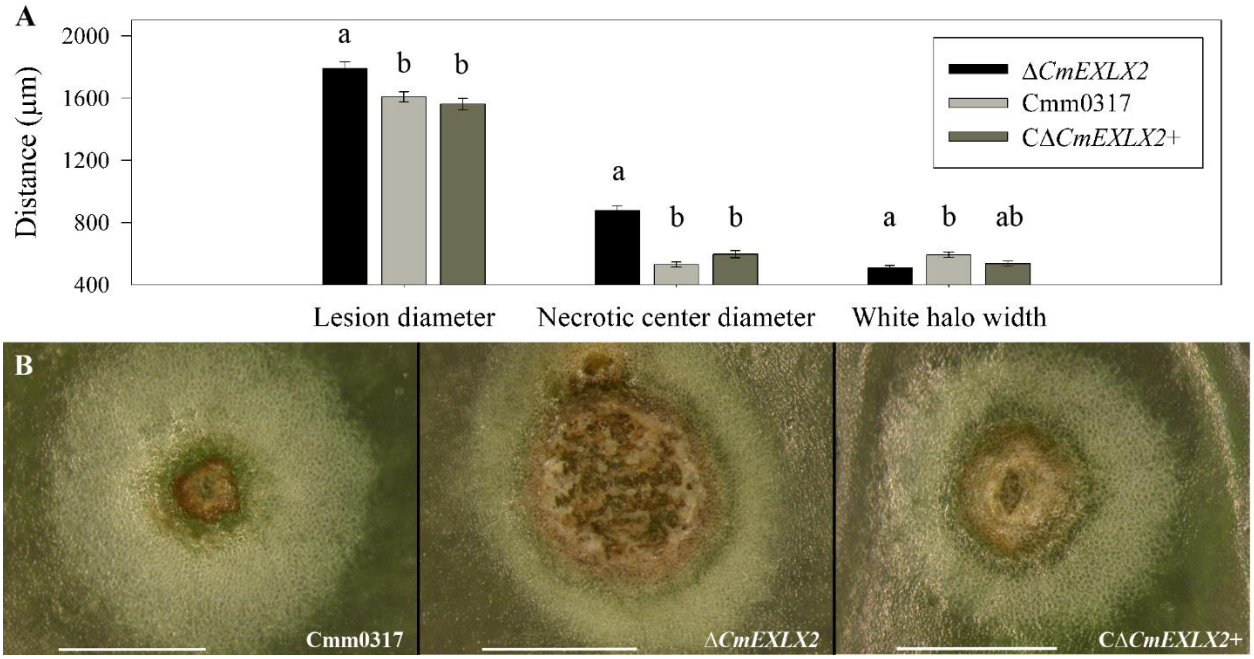

Figure 3.7. Influence of *Clavibacter michiganensis* subsp. *michiganensis* expansin mutant Δ*CmEXLX2* on tomato fruit lesion development (A) Lesion sizes on tomato fruit (n=100) inoculated with the three *C. michiganensis* subsp. *michiganensis* strains. Fruit lesion distances followed by the same letter are not significantly different. Significant differences among treatments for mean lesion size were tested with PROC GLIMMIX (P<0.05) followed by Tukey-Kramer posttest (P<0.01). (B) Representative images of tomato fruit lesions are shown below: Cmm0317 (wildtype), Δ*CmEXLX2* (expansin mutant), and CΔ*CmEXLX2*$^{+}$ (complement). Scale bar represents 1 mm.

Symptom development of tomato seedlings infected with the *Rs* Δ*RsEXLX* mutant. Since plant-like expansins have been acquired by many xylem-dwelling plant pathogenic bacteria, we hypothesized that plant-like expansins would also affect the virulence of another xylem

pathogen, *Rs*. Because *Rs* infects host plants via the roots before systemic colonization of the xylem, we used two inoculation methods to separate possible roles of expansin in *Rs* virulence. Soil-drench inoculation of unwounded plants mimics the natural route of infection while the cut-petiole technique bypasses root infection by introducing the pathogen directly into the xylem. In contrast to *Cmm*, the *Rs* expansin mutant had wildtype level virulence when directly introduced into tomato stems (P=0.106), but was significantly reduced in virulence following soil-drench inoculation (P=0.0465) (Figure 3.8). By 14 DPI, average symptom incidence was only 56% for the *Rs* expansin mutant, but 87% and 89% for the wildtype and complement, respectively.

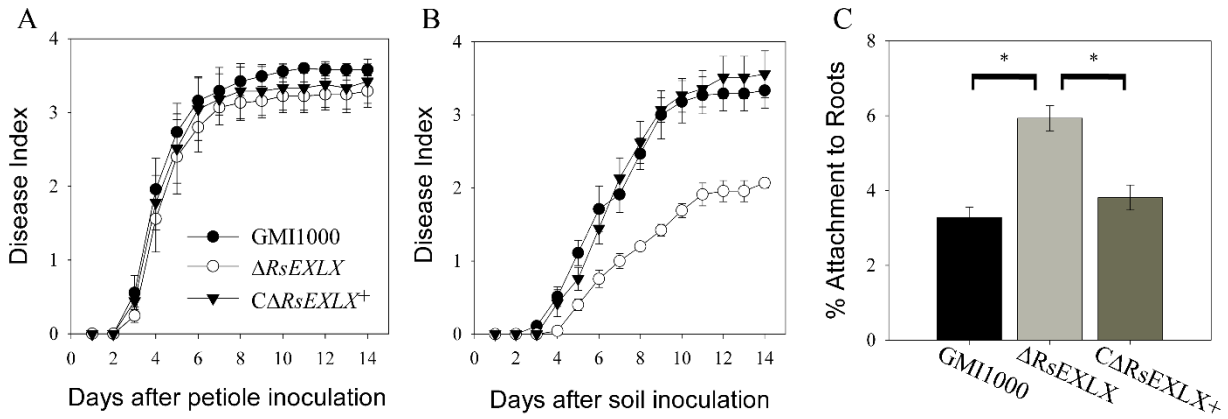

Figure 3.8. Virulence and root attachment of *Ralstonia solanacearum* expansin mutant. (A) Plants were directly inoculated into stem vasculature by placing 1000 CFU onto the surface of a freshly-cut leaf petiole (P=0.1056, repeated measures ANOVA), or (B) naturalistically inoculated by drenching the soil of tomato plants with ~$5x10^8$ CFU bacteria /g soil (P=0.0465, repeated measures ANOVA). Experiments were repeated 3 times with total N=45 plants per strain. (C) *R. solanacearum* strains were incubated for 2h with sterile tomato seedling roots. Percent of bacteria attached to roots after washing was measured by serial dilution plating. * indicates P<0.0001 by ANOVA.

Expansins influence *Rs* but not *Cmm* attachment. The virulence defect of the *Rs* expansin mutant following soil-drenching but not direct xylem inoculation suggested that the *Rs* expansin contributes to early infection. To investigate this early stage of infection, we compared bacterial

attachment on emergent tomato seedling roots following a 2 h incubation. The *Rs* expansin mutant hyper-attached to roots compared to wildtype ($P<0.0001$). After bacterial suspensions were incubated with tomato roots, 6% of the *Rs* expansin mutant population attached to roots compared to 3.3% and 3.8% of the wildtype and complement populations, respectively (Figure 3.8). In contrast, there were no differences in root attachment between the *Cmm* strains ($P=0.258$). All three *Cmm* strains were capable of root attachment, albeit weakly, with an average of 4 CFU/root with an overall range of 0-25 CFU/root (Table 3.4).

Table 3.4. Attachment of *Clavibacter michiganensis* subsp. *michiganensis* strains colonizing tomato roots following a 2 hour incubation.

Strain	CFU/Root[†]
Cmm0317	5.17 [A]
Δ*CmEXLX2*	5.96 [A]
CΔ*CmEXLX2*$^{+}$	1.50 [A]
Water	0.0

[†]The mean bacterial population recovered from tomato roots inoculated with *C. michiganensis* subsp. *michiganensis* and incubated for 2 hours at room temperature. The experiment was performed three times. Significant differences among strains were tested using ANOVA ($P<0.05$) followed by Tukey's studentized range posttests ($P<0.05$). Bacterial populations followed by the same superscript are not significantly different.

To determine if the attachment phenotype was root specific or biofilm related, *Rs* and *Cmm* strains were analyzed for bacterial attachment *in vitro*. No differences in biofilm formation were detected between *Rs* strains ($P=0.111$) or *Cmm* strains ($P=0.117$). Interestingly, little to no biofilm was observed with *Cmm* strains when grown in xylem mimicking medium (Hiery et al. 2013) or LB media (Figure 3.9). However, *Cmm* strains grown in pure tomato xylem sap displayed high levels of biofilm formation with no difference between Δ*CmEXLX2* (mean $OD_{590\ nm}=0.88$) and wildtype (mean $OD_{590\ nm}=0.94$).

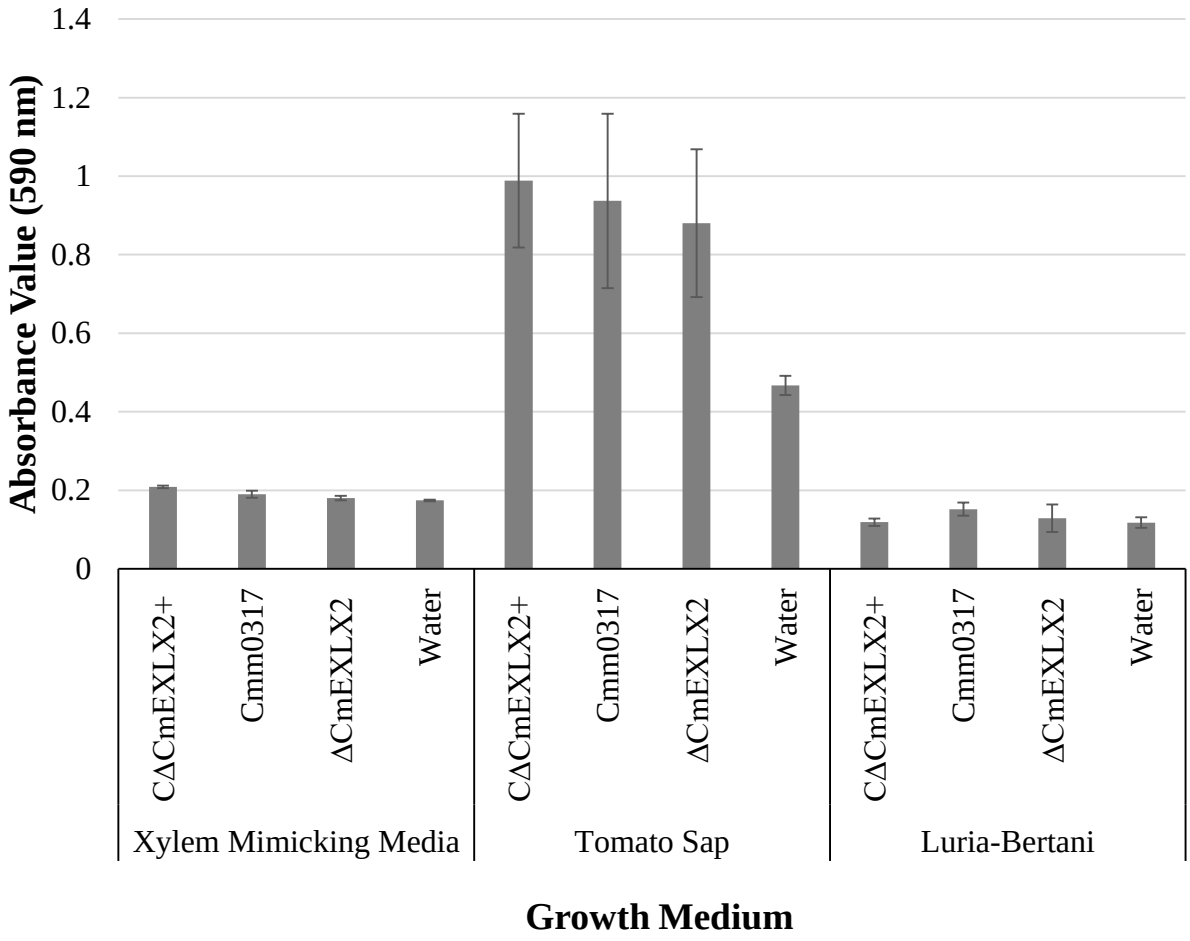

Figure 3.9. *In vitro* attachment of *Clavibacter michiganensis* subsp. *michiganensis* Cmm0317 (wild-type), Δ*CmEXLX2* (expansin mutant), and CΔ*CmEXLX2*$^{+}$ (complement) in xylem mimicking media, tomato sap, and nutrient-rich LB medium.

DISCUSSION

Functional plant-like bacterial expansins were first confirmed in *B. subtilis*, yet identified within a wide array of plant-associated bacteria (Kerff et al. 2008; Nikolaidis et al. 2014). However, the role of non-chimeric bacterial expansins has not been investigated for any phytopathogenic bacteria. We found the loss of non-chimeric bacterial expansins had different effects on two vascular pathogens of tomato, *Cmm* and *Rs*, which independently acquired plant-

like expansins and appear to have selectively modified their functions to have divergent roles in plant pathogenesis.

Disruption of *CmEXLX2* had no observable impact on *in vitro* growth rates or recognition by *M. jalapa*, a non-host. Similarly, an *Rs* expansin mutant behaved like its wildtype parent strain *in vitro*. To ensure that bacterial autolysis did not influence colonization/infection, autolytic rates were explored in *Cmm* and *Rs* strains. We found that *Cmm* and *Rs* expansin mutants displayed wildtype-levels of autolysis. In contrast, a *B. subtilis* expansin mutant had reduced autolysis despite the BsEXLX1 expansin peptide lacking lytic activity against peptidoglycan (Kerff et al. 2008).

Following inoculation with *Cmm* strains, the *Cmm* expansin mutant induced greater disease severity on tomato seedlings as characterized by a rapid onset of unilateral wilting, increased necrosis, and larger population sizes. Although all three *Cmm* strains produced typical stem lesions at the site of inoculation, only the *Cmm* expansin mutant produced numerous atypical necrotic lesions on the vasculature of distant petioles. Necrosis appeared to be confined to the vasculature of infected tomato plants with healthy tissue surrounding the lesions until later stages of disease. The quick onset of symptoms by the *Cmm* expansin mutant was not correlated with increased intra- or intervascular spread because EGFP-expressing isolates revealed similar rates of vascular infection both above and below the site of inoculation. Similarly, the *Cmm* expansin mutant caused larger and more necrotic tomato fruit lesions. Fruit infected with the *Cmm* expansin mutant had larger, more blistered, and more necrotic lesions with less noticeable 'white halos'. Some *Cmm* expansin mutant fruit lesions were atypical and developed necrosis, but completely lacked the characteristic 'white halo' commonly associated with the disease. Fruit

lesions are a relatively unexplored disease symptom associated with bacterial canker, and the significance of the 'white halo' that surrounds the lesion remains unknown.

In planta bacterial populations were significantly different between *Cmm* strains with the expansin mutant having larger populations 1 cm above the site of inoculation. However, differences in population size between *Cmm* strains dissipated as higher regions of the stem were colonized; and at 10 cm above the inoculation site no significant differences existed between the three strains. This suggests that the more established Δ*CmEXLX2* populations close to the inoculation site were proliferating at higher rates relative to the wildtype and complemented mutant. Because the *Cmm* expansin mutant grew like wildtype *in vitro,* the mutant's increased *in planta* population size was likely due to the increased necrosis induced by the mutant. The established Δ*CmEXLX2* populations near the inoculation site may degrade vascular tissue more quickly, which likely released plant-derived nutrients into the nutrient-poor xylem. The greater rates of necrosis observed on the plant and fruit may be directly associated with the increased availability of plant-derived nutrients and subsequent influx of bacterial growth.

The molecular mechanism underlying the increased necrosis of the *Cmm* expansin mutant is unknown. One hypothesis is that the CmEXLX2 expansin competes with the numerous *Cmm* cell-wall degrading enzymes for unique binding sites in the plant cell wall. In support of this hypothesis, Georgelis et al. (2012) demonstrated that the *B. subtilis* BsEXLX1 expansin competes with type-A cellulose-binding modules for binding sites in crystalline cellulose. Binding competition was suggested to result from the similar features shared between BsEXLX1 (domain D2) and other type-A cellulose-binding modules (Georgelis et al. 2012). Furthermore, plant α-expansins induce rapid cell wall extension (<1 minute) with no lasting structural changes, in contrast to other cell wall modifying enzymes like pectinases, cellulases, and hemicellulases

(McQueen-Mason et al. 1992; Yuan et al. 2001). Without competition from the *Cmm* expansin, the efficiency of the *Cmm* cell-wall degrading enzymes may have increased, resulting in major structural changes and the breakdown of the plant cell wall (i.e. necrosis).

The increased necrosis caused by the *Cmm* expansin mutant likely poses a fitness cost at another life cycle stage, such as root colonization or seed infection. We investigated the role of the expansin in root attachment by *Cmm*, but we did not find any differences between strains. The non-chimeric *Cmm* expansin investigated in this study shares only 56% identity with the chimeric *Cmm* expansin (Figure 3.10). Interestingly, previous studies have shown that disrupting chimeric expansins reduce disease symptoms (Jahr et al. 2000; Laine et al. 2000; Ingel et al. 2015). This reduction of disease symptoms, observed with *X. fastidiosa* and *Cmm* infections, could be attributed to novel functions associated with chimeric expansins since they co-evolved with an attached endoglucanase (glycosyl hydrolase family 5-GH5), independent of the non-chimeric expansin (Nikolaidis et al. 2014; Ingel et al. 2015; Jahr et al. 2000).

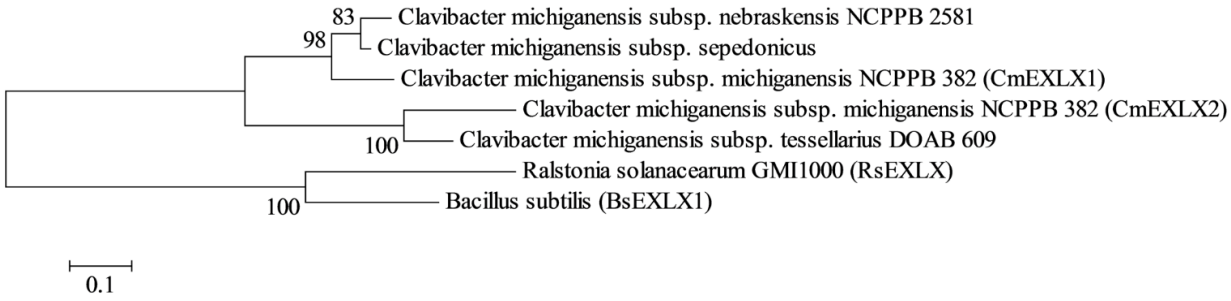

FIGURE 3.10. Maximum-likelihood phylogenetic tree for bacterial expansins represented by *Clavibacter michiganensis*, *Ralstonia solanacearum*, and *Bacillus subtilis*. Alignment gaps were excluded, and the total number of sites used was 188 with 1000 repetitions. Bootstrap values are shown at the nodes if greater than 50%. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site (Tamura et al. 2011).

Due to the increased symptom development observed in *Cmm* infections, it was important to determine if other non-chimeric bacterial expansins functioned in a similar manner during vascular pathogenesis. The Gram-negative vascular tomato pathogen *Rs* was selected for comparative studies. Unlike *Cmm*, the *Rs* expansin mutant had wildtype-level virulence when directly introduced into the tomato vasculature. However when introduced into the soil (a more natural route of infection), the *Rs* expansin mutant caused significantly less disease than wildtype. The reduced virulence following soil-drenching inoculation could be due to the *Rs* expansin mutant's hyper-attachment to tomato roots, which could impair the mutant's ability to enter and colonize the root vasculature.

Bacterial non-chimeric expansins appear to have undergone intense selective pressures to adapt to their hosts. Epidemiologically, *Cmm* is characterized as a foliar pathogen that accesses xylem vessels through wounds or natural openings, explaining how CmEXLX2 appears to function in a xylem-specific manner with no major role in bacterial attachment. Conversely, the soil-borne pathogen *Rs* colonizes xylem vessels after root infection, but the *Rs* expansin mutant hyper-attached to the roots impairing colonization. Lastly, the saprophytic root-colonizing bacterium *B. subtilis* requires a non-chimeric expansin to attach to maize roots (Kerff et al. 2008). These results suggest three unique and contrasting phenotypes attributed to non-chimeric bacterial expansins (Table 3.5). This divergence in function is not novel to bacterial expansins, but is represented throughout the plant kingdom as unique cell-specific expansins are responsible for modulating distinct aspects of plant cellular growth (Cosgrove 2000, 2015). For example, *Arabidopsis* maintains at least 24 α-expansins with a variety of putative roles, and *Zinnia elegans* has a subset of three xylem-specific expansins that vary in temporal and spatial expression (Cosgrove 2000; Im et al. 2000). The targets of plant and microbial expansins remain unknown,

but differences in expansin structure and isoelectric points could influence activity and substrate differentiation (Pastor et al. 2015).

Only a subset of plant-associated bacteria possess expansin genes, including saprophytes and plant pathogens, while other plant-associated microbes such as *Pseudomonas* or symbionts like *Rhizobium* lack expansins (Nikolaidis et al. 2014). The conservation of this plant xenolog in vascular-colonizing phytopathogenic bacteria does not imply that the function is xylem limited. Instead increased necrosis appearing on the tomato fruit and the vasculature with *Cmm* infections, leads us to speculate that vascular-inhabiting bacterial pathogens horizontally acquired and maintained the expansin gene to exploit the acidic microenvironment of the xylem. Expansins are pH-dependent with an optimal pH range of 4.5 – 6.0, which overlaps the pH range of xylem fluid (pH~5.5) and tomato fruit tissue (pH~4.4) (Jones 1999; Cosgrove 2005; Urrestarazu et al. 1996; Bollard 1960). Perhaps the acidic microenvironment of the xylem provides the optimal environment for expansin activity, which is not observed in the neutral apoplastic fluid. However, putative chimeric expansins are present in several non-vascular plant-associated microbes such as the non-vascular *Xanthomonas* species: *X. oryzae* pv. *oryzicola*, *X. translucens*, and *X. campestris* pv. *raphani* (Ryan et al. 2011; Nikolaidis et al. 2014). *Streptomyces* appears to be the only currently known genus that contains non-vascular phytopathogenic bacteria possessing bacterial expansins. However, some phytopathogenic *Streptomyces* sp., such as *S. acidiscabies* can tolerate acidic soils with minimum growth pH values ranging between 4 and 5.5 depending on the strain (Lambert and Loria 1989b, 1989a). Nikolaidis et al. (2014) highlighted how *S. acidiscabies* appears to be the most recent microbe to acquire a plant-like expansin because the sequence has greater similarities to other plant expansins than any known microbial expansin (Nikolaidis et al. 2014).

TABLE 3.5. Overview of bacterial phenotypes associated with non-chimeric bacterial expansins

Bacteria	Lifestyle	Primary route of colonization	Non-chimeric expansin	% identity[†]	Expansin mutant phenotype		Reference
					Disease symptoms	Root attachment	
Clavibacter michiganensis subsp. *michiganensis*	Vascular phytopathogen	Foliar	*CmEXLX2*	100%	Increase	No difference	This study
Ralstonia solanacearum	Vascular phytopathogen	Root	*RsEXLX*	35%	Decrease	Hyper-attach	This study
Bacillus subtilis	Saprophyte	Root	*BsEXLX1*	35%	n/a	Decrease	(Kerff et al. 2008)

[†]Percent amino acid identify of expansin D1/D2 domains, relative to the non-chimeric *Clavibacter michiganensis* subsp. *michiganensis* expansin *CmEXLX2*.

The independent horizontal acquisition of a plant gene and its subsequent prokaryotic-specific adaptations highlight the dynamic nature of plant-bacterial interactions. Appropriation of plant expansins may have allowed microbes to manipulate or possibly mimic the biological processes of host tissue. Within *Cmm*, CmEXLX2 influences vascular and fruit necrosis leading to larger *in planta* populations and symptom progression within tomato. In contrast, RsEXLX appears to contribute to root infection, possibly by modulating root attachment. Future research could further characterize the role RsEXLX plays in *Rs* root infections, and elucidate the targets and molecular functions of these diverse bacterial plant-like expansins.

MATERIALS & METHODS

All bacterial strains/plasmids and primers used in this study are listed in Table 3.6 and 3.7, respectively.

Table 3.6. Bacterial strains and plasmids used in this study

Strain or Plasmid	Description[†]	Reference/Source
Strains		
C. michiganensis subsp. *michiganensis*		
Cmm0317	Wild-type virulent New York strain	(Tancos et al. 2015)
Δ*CmEXLX2*	Cmm0317 mutant strain lacking *CmEXLX2*	This study
CΔ*CmEXLX2*$^{+}$	Δ*CmEXLX2* transformed with pHNExpA (complement)	This study
Escherichia coli		
Zymo 5α (DH5α)	Cloning strain	Zymo Research
E.coli ER2925	*dam* and *dcm* methylation-negative strain	New England Biolabs
R. solanacearum		
GMI1000	Wild-type virulent strain isolated from tomato	(Salanoubat et al. 2002)
Δ*RsEXLX*	GMI1000 mutant with unmarked *RsEXLX* deletion	This study
CΔ*RsEXLX*$^{+}$	Δ*RsEXLX* transformed with pMiniTn7-RSc0818comp (complement)	This study
Plasmids		
C. michiganensis subsp. *michiganensis*		
pGEM-T Easy	Cloning vector; Amp^{r}; 3 kb	Promega
pHN216	*E. coli–Clavibacter* shuttle vector; Gm^{r} Nm^{r}; 13.8 kb	(Laine et al. 1996)
pGnR-BsiWI	pGEM-T Easy:Gm^{r} cassette (from pHN216); Amp^{r} Gm^{r}; 3.8 kb	This study
pGCMEβ	pGEM-T Easy:*CmEXLX2*; 3.6 kb	This study
pGCMEβGM	pGEM-T Easy:*CmEXLX2*::Gm^{r} cassette; Amp^{r} Gm^{r}; 4.3 kb	This study
pIDT-FLExpA	IDT vector:Full length *CmEXLX2* and promoter; Amp^{r}; 3 kb	IDT
pHNExpA	Full length *CmEXLX2* cloned into pHN216; Nm^{r}; 13.1 kb	This study
pK2-22	eGFP-expressing plasmid; Nm^{r}; 13.5 kb	(Chalupowicz et al. 2012)
R. solanacearum		
pUFR80	Cloning vector; Kan^{r} Suc^{s}; 7.8 kb	(Castañeda et al. 2005)
pUFR80-RSc0818KO	pUFR80:Unmarked Δ*RsEXLX* cassette; Kan^{r} Suc^{s}; 9.4 kb	This study

pUC18t-MiniTn7t(Gm)	Wide host range complementation vector; Gm^r; 4.6 kb	(Choi et al. 2005)
pMiniTn7-RSc0818comp	pUC18t-MiniTn7t(Gm):full length *RsEXLX* ; Gm^r; 6.1 kb	This study

[†]Gm^r, Gentamicin acetyltransferase; Nm^r, aminoglycoside (neomycin) phosphotransferase; Amp^r, β-lactamase; Kan^r, aminoglycoside 3'-phosphotransferase; Suc^s, levansucrase

Table 3.7. Oligonucleotides used in this study

Name	Sequence (5'-3')[†]	Target	Purpose	Locus Tag	Tm (°C)	Amplicon size (bp)	Reference
ExpA	F:GAGGACGACGAACGGCAACTG R:TGCTGCAGCCTCATCGGGTC	*CmEXLX2*	Cloning	CMM_1480	62	533	This study
GnR-BsiWI	F:ACAGCGCGTACGACCCAGTTGACATAAGCCTGTTCG R:ACAGCGCGTACGCGGCTTGAACGAATTGTTAGGTGG	Gentamicin acetyltransferase	Cloning	NA	62	778	This study
Gnx	F:CAGTTGACATAAGCCTGTTCG R:GCTTGAACGAATTGTTAGGTGG	Gentamicin acetyltransferase	Verification	NA	58	768	This study
Cmm1481	R:GGTCGGGCGAGTCGGAG	*CmEXLX2* downstream	Verification	CMM_1481	68	1474	This study
1479	F:GACCGTCGAGCAGATCGCC	*CmEXLX2* upstream	Verification	CMM_1479			
ExpANew	F:TGCCACGAGTGCGTGCACG R:GTGGTGTCCGTGACGCGCAC	*CmEXLX2*	Verification	CMM_1480	64	320	This study
Multiple cloning site	T7:TAATACGACTCACTATAGGG SP6:ATTTAGGTGACACTATAG	pGEM-T Easy	Sequencing	NA	50	NA	
ExpA-1479	R:CAGTTGCCGTTCGTCGTCCTC	pHNExpA	Sequencing	CMM_1480	61	NA	This study
ExpA-UP2	F:GCCCGCGTGTCCGGCTACG	Upstream of *CmEXLX2* insertion	Sequencing	CMM_1480	62	NA	This study
HI-GC-Insert	F:ATAGGGGTTCCGCGCACATTTCC R:CATCGGAGCGGGGTTCATGTGGCT	*CmEXLX2* insert in pHNExpA	Verification/sequencing	NA	67	1124	This study
GyrB	F:ACCGTCGAGTTCGACTACGA R:CCTCGGTGTTGCCSARCTT	DNA gyrase, subunit B	RNA expression	CMM_0006	60	525	(Zaluga et al. 2013; Richert et al. 2005)
RSc0818up	F:*GATATCTGAATTCGTCG*ACAAGAGCTACTCGCCGCTGT R:*CCAGAGCTGC*GACCGCAATCCATCGCCATTT	*RsEXLX* upstream	Cloning	RSc0818	60	713	This study
RSc0818dwn	F:*AAAATCCCTT*GCGGTGGTGGGTGATCAG R:*GAGGTACCGGGCCCA*GCAACCTATTCGCTACGG	*RsEXLX* downstream	Cloning	RSc0818	60	873	This study
RSc0818com	F:*CAGGAATTCCTCGAGA*TCAGCAACAATGCGCCAA R:*GAGGTACCGGGCCCA*GCAACCTATTCGCTACGG	*RsEXLX* and promoter	Cloning	RSc0818	60	1511	This study
RSc0818int	F:ATCACACCTTCCCAGTGGTG R:GTGTGCAATCCTTGAGCAGC	*RsEXLX* internal	Verification	RSc0818	60	186	This study
M13	F:CCCAGTCACGACGTTGTAAAACG R:AGCGGATAACAATTTCACACAGG	pUFR80	Sequencing	NA	58	N/A	

SeqTn7	F:GTTGACAAAGGGAATCAGGGGATC R:CACTTATCTGGTTGGCCTGCAAG	pUC18t-MiniTn7t(Gm)	Sequencing	NA	58	NA	This study

†Underline indicates restriction enzyme cut site and italics indicates overlaps for Gibson assembly

Bacterial strains and growth conditions. Bacterial strains and plasmids used in this study are listed in Table 3.6. The *Cmm* strain (Cmm0317) used in the present study was a virulent New York field strain, which naturally lacked the *celA* gene (chimeric expansin *CmEXLX1*) (Tancos et al. 2015). Depending on the assay, *Cmm* isolates were incubated for 3-4 days at 27°C in Luria-Bertani (LB) (Miller 1972), SB (Kirchner et al. 2001; Stork et al. 2008), or D2ANX media (Hadas et al. 2005). When required, LB medium was supplemented with the antibiotics gentamicin (40 µg/ml), kanamycin (100 µg/ml), or ampicillin (100 µg/ml) (Fisher Scientific; Pittsburgh, PA).

The phylotype I *Rs* strain GMI1000 was used in this study. *Rs* was routinely grown in casamino acid, peptone, glucose media (CPG) or Boucher's minimal media (BMM) with 20 mM glucose at 28 °C (Boucher et al. 1985). When required, media were supplemented with the antibiotic gentamicin (25 µg/ml) (Fisher Scientific; Pittsburgh, PA). All *Cmm* and *Rs* isolates were stored in 35% glycerol at -80°C.

Construction of plasmids pGCMEβGM, pHNExpA, pUFR80-RSc0818KO, and pMiniTn7-RSc0818comp. The *Cmm* expansin gene, *CmEXLX2*, was amplified with primers ExpA-F/R (Table 3.7) to generate a 533-bp PCR product, which was cloned into a pGEM-T Easy vector (Promega Corp, Madison, WI) yielding pGCMEβ. A gentamicin resistance cassette (produced from pHN216 (Laine et al. 1996)) with artificial *Bsi*WI restriction sites was cloned into a pGEM-T Easy vector to yield pGnR-BsiWI, and subsequently excised as a 779 bp fragment and ligated into the single *Bsi*WI site of *CmEXLX2* in pGCMEβ to yield pGCMEβGM using *E.coli* Zymo 5α (Zymo Research Inc., Irvine, CA). The pGCMEβGM was subsequently transferred into *dam*$^-$ and *dcm*$^-$ *E.coli* ER2925 (New England Biolabs, Ipswich, MA) in order to increase transformation efficiency in *Cmm* (Table 3.6) (Kirchner et al. 2001). For complementation

studies, the native promoter and full-length *CmEXLX2* gene (based on *Cmm* strain NCPPB382) with artificial *Eco*R1 cut sites was synthesized from Integrated DNA Technologies (IDT, Inc. Coralville, IA) to yield pIDT-FLExpA (Table 3.6). *CmEXLX2* was excised from pIDT-FLExpA using *Eco*R1 and cloned into compatible sites within pHN216 (Laine et al. 1996), replacing the aminoglycoside (neomycin) phosphotransferase gene and producing pHNExpA.

The deletion vector pUFR80-RSc0818 was generated by Gibson assembly (Gibson 2011; Gibson et al. 2009). The 713 bp region upstream of *RsEXLX* was amplified with RSc0818upF/R, and the 873 bp region downstream of *RsEXLX* was amplified with RSc0818dwnF/R. The PCR products were assembled into *Hind*III-digested pUFR80. The complementation vector pMiniTn7-RSc0818comp was generated by Gibson assembly. The 1.5 kb region encompassing the native promoter and full length *RsEXLX* gene was amplified with RSc0818F/R and assembled into *Hind*III-digested pUC18t-MiniTn7t(Gm). After assembly, constructs were transformed into *E. coli* Top10 (Life Technologies) and screened by PCR, diagnostic restriction digest, and sequencing.

Transformation and isolation of mutants. DNA of pGCMEβGM, pHNExpA, and pK2-22 (Chalupowicz et al. 2012) was extracted from *dam*$^{-}$ and *dcm*$^{-}$ *E.coli* ER2925 with E.Z.N.A Fastfilter plasmid midi kit (Omega Bio-Tek; Norcross, GA). Competent cells were prepared and transformed as previously described by Stork et al. (2008) and Tancos et al. (2013), respectively. The vector pGCMEβGM was electroporated into the wild-type *Cmm* strain 0317 (Cmm0317) yielding the *CmEXLX2* mutant (Δ*CmEXLX2*). Sequencing of the upstream (CMM_1479) and downstream (CMM_1481) genes in the mutant strain Δ*CmEXLX2* confirmed that the cassette was correctly inserted. The wild-type *CmEXLX2* expressing vector, pHNExpA, was subsequently electroporated into the mutant strain Δ*CmEXLX2*, yielding the complemented

mutant C∆*CmEXLX2*$^{+}$. Putative *Cmm* mutants were grown on LB medium amended with gentamicin (40 µg/ml) and/or kanamycin (100 µg/ml) and selected following 3-4 days growth at 27°C.

The unmarked *Rs* ∆ *RsEXLX* mutant was generated by natural transformation with pUFR80-RSc0818KO as described in (Boucher et al. 1985; Lowe et al. 2015). Briefly, transformants were plated on kanamycin to select for vector integration at the *RsEXLX* locus. Then kanamycin-resistant clones were counter-selected on 5% w/v sucrose to select for loss of the pUFR80 vector backbone by homologous recombination. Sucrose resistant clones were restruck on unamended media. PCR screening with RSc0818intF/R primers and GoTaq Green PCR mastermix (Promega, Madison, WI) was used to confirm the loss of the *RsEXLX* gene.

PCR analysis and RNA expression. Confirmation of plasmid constructs and chromosome integrations were confirmed with gene-specific PCR and sequencing (Table 3.7). Genomic *Cmm* DNA was extracted with the MasterPure Gram-Positive DNA purification kit (Epicentre, Madison, WI) according to the manufacturer's instructions. Amplification was performed in a C1000 Touch Thermal Cycler (Bio-Rad Laboratories, Inc., Hercules, CA) using EmeraldAmp GT PCR Master Mix (Takara Bio Inc., Otsu, Shiga, Japan) or Advantage-GC 2 Polymerase Mix with a final GC-Melt concentration of 1.0 M for high GC sequences (Clontech Lab. Inc., Mountain View, CA), according to the manufacturer's instructions. Extracted total DNA was used at approximately 50 ng per reaction with 10 µM each of forward and reverse primers (Table 3.7). PCR (EmeraldAmp GT PCR Master Mix) was performed in 25 µl reactions with the following parameters: initial preheat for 3 min at 95°C, 35 cycles at 95°C for 15 sec, a primer pair specific annealing temperature for 30 sec (Table 3.7), 72°C for 1 min, a final extension at 72°C for 5 min, and held at 12°C. PCR (Advantage-GC 2 Polymerase Mix), used for primers

Cmm1481, 1479, and HI-GC-Insert F/R, was performed in 25 µl reactions with the following parameters: initial preheat for 3 min at 94°C, 35 cycles at 94°C for 15 sec, 68°C for 1.5 min, a final extension at 68°C for 5 min, and held at 12°C. PCR products were purified with a DNA Clean & Concentrator – 25 kit (Zymo Research Inc., Irvine, CA) according to the manufacturer's instructions. The cleaned PCR amplicons were sequenced on a 3730XL DNA Analyzer (Applied Biosystems) at the Cornell University Life Sciences Core Laboratories Center.

RNA was extracted from 6 ml of *Cmm* suspension grown in LB medium to an OD_{600nm}= 1.4. Total RNA was extracted using a ZR Fungal/Bacterial RNA MiniPrep kit (Zymo Research Corporation, Irvine, CA). Extraction was followed by an additional DNase treatment using TURBO DNA-free DNase (Fisher Scientific; Pittsburgh, PA). Quantity and quality (260/280 ratio) of DNase-treated RNA was determined with a NanoDrop ND 1000 (NanoDrop Technologies, Wilmington, DE). Reverse-transcription was performed with RNA to cDNA EcoDry Premix (random hexamers) (Clontech Laboratories, Inc. Mountain View, CA) using 300 ng of RNA per reaction. The resultant cDNA ($1/10^{th}$ of the reverse-transcription reaction volume) was used in a 25 µl PCR reaction using EmeraldAmp GT PCR Master Mix (Takara Bio Inc.) with ExpANew-F/R or GryB-F/R primers (Table 3.7). To ensure no DNA remained in the RNA, controls were performed with pure RNA (100 ng/reaction). PCR products were electrophoresed on 1% gels at 90V for 45 minutes, followed by staining with GelRed (Biotium Inc., Hayward, CA).

Assessing *in vitro* growth and hypersensitive response assays. *In vitro* growth rates for Δ*CmEXLX2* and wild-type were compared in LB medium over a 48 hour growing period. Additionally, Δ*CmEXLX2*, wild-type, and the complemented mutant growth rates were compared in pure tomato xylem sap. In order to harvest tomato xylem sap, tomato seedlings (*Solanum*

lycopersicum), cultivar Mountain Fresh Plus, were grown in a Fafard professional formula growing mix (Sun Gro Horticulture, Agawam, MA) with a 14-hr light/ 10-hr dark photoperiod in the greenhouse. The tomato seedlings were fertilized weekly with Peters 15-5-15 Cal-Mag mixture (Everris NA, Inc.; Netherlands). Tomato xylem sap was harvested from 6-8 week old tomato seedlings (n=50). Stems were cut approximately 10-cm above the soil, placed horizontally (at a downward slant) for gravity-assisted dripping, after the initial 5 minutes the stems were wiped clean to avoid possible phloem sap contamination, and then xylem sap was collected in 15-ml sterile tubes (Chalupowicz et al. 2012; Hiery et al. 2013). Sap was collected and pooled every 1-2 hours and refrigerated until minimal sap continued to flow (approximately 5-7 hours later). Pooled collected sap was filter-sterilized through a 0.22 µm PES membrane 500-ml filter system (Corning Inc. Corning NY) and frozen in aliquots at -20°C.

Isolates for *in vitro* growth assays were initially grown in LB medium for 28 hours and then adjusted to OD_{600nm}= 0.6. The bacterial suspensions (1 ml) were pelleted at 4602 x g for 4 minutes, washed and centrifuged, resuspended in 1 mL of distilled water, and inoculated (50 µl) into a 250 ml flask containing 50 ml of fresh LB medium or pure tomato xylem sap (three replicates/strain/medium). Cultures were incubated at 27°C with shaking at 160 rpm, and cells (800 µl) were quantified at OD_{600nm} using a spectrophotometer (Eppendorf, Hamburg, Germany) every 4 hours for 48 hours (Xu et al. 2010). The mean area under the *in vitro* growth curve was calculated for both LB medium and pure tomato xylem sap. Significant differences among *in vitro* growth rates were tested with ANOVA ($P<0.05$) followed by Tukey's HSD (honestly significant difference) posttest ($P<0.05$) using SAS v. 4.3 (SAS Institute, Cary, NC).

Cmm strains were tested for a hypersensitive response (HR) in *Mirabilis jalapa* (four O'clock plants). *M. jalapa* plants were grown in a Cornell potting mix (comprised of peat,

perlite, and vermiculite in a 4:1:1 ratio), fertilized with Smart-Release Osmocote (Scotts Miracle-Gro Company, Marysville, OH), with a 14-hr light/ 10-hr dark photoperiod in the greenhouse. *Cmm* strains were grown for 24-32 hours in LB medium (gentamicin and/or kanamycin were added for Δ*CmEXLX2* or the complemented mutant) while shaking at 160 rpm in 125 ml flasks at 27°C, adjusted to OD_{600nm}= 0.8 (10^8 CFU/ml), pelleted at 3041 x g for 7 minutes, washed and centrifuged, and resuspended in sterile water. The bacterial suspensions were syringe-injected into the abaxial surface of the expanded *M. jalapa* leaf using a needleless 10-ml syringe.

In vitro growth of *Rs* strains was determined in CPG and BMM broth. Strains were grown overnight in CPG broth, washed twice in water. Then bacterial suspensions were adjusted to an $OD_{600\ nm}$=0.1 in CPG or BMM and 200 µl of the cell suspensions were added to individual wells (n=3/strain) of a 96-well Falcon tissue culture plate (Corning Inc., Corning, NY). The plate was incubated at 28 °C with shaking in a BioTek microplate reader (Winooski, VT). Bacterial density was measured at 600 nm every 15 minutes for 24 hours. The mean area under the *in vitro* growth curve was calculated for both CPG and BMM broth. Significant differences among *in vitro* growth rates were tested with ANOVA ($P<0.05$) followed by Tukey's HSD (honestly significant difference) posttest ($P<0.05$) using SAS v. 4.3 (SAS Institute, Cary, NC).

Cell autolysis assays. *Cmm* isolates were grown in LB broth (gentamicin and/or kanamycin were added for Δ*CmEXLX2* or the complemented mutant) as described above for 24 hours, bacterial suspensions were pelleted at 3041 x g for 6 minutes, washed twice with 1x PBS and centrifuged, and resuspended in 1x PBS. The bacterial suspensions were adjusted to an OD_{600nm}= 0.8 with 1x PBS and pelleted at 4602 x g for 4 minutes and resuspended in 2 mL of 1x PBS with 0.02% SDS (pH=7.0). *Cmm* suspensions (200 µl) were added to the individual wells (n=4/strain) of a 96-well Falcon tissue culture plate (Corning Inc., Corning, NY). The plates

were placed in a BioTek micoplate reader (Winooski, VT) at 28°C for 10 hours with the bacterial suspension being measured at 590 nm every 30 minutes. The experiment was performed three times for a total of 12 absorbance readings/strain at each timepoint. Significant differences among treatments were tested with PROC GLIMMIX ($P<0.05$) followed by Tukey-Kramer posttest ($P<0.05$) using SAS v. 4.3 (SAS Institute, Cary, NC).

To determine if the *Rs* expansin mutant differed in cell lysis compared to the wild-type strain, cells from overnight CPG cultures of GMI1000, Δ*RsEXLX*, CΔ*RsEXLX* $^{+}$ were collected by centrifugation, washed twice in PBS and adjusted to A_{600nm}=1.0. Cells were pelleted again and resuspended in PBS+0.02% SDS. Bacterial suspensions (200 μl) were seeded into a 96-well microtiter plate and absorbance at 600 nm was monitored every 30 minutes for 12 hours without agitation using a Biotek HT plate reader (Biotek, Winooski, VT). The experiment was repeated three times, each with three technical replicates. Significant differences among treatments were tested with PROC GLIMMIX ($P<0.05$) followed by Tukey-Kramer posttest ($P<0.05$) using SAS v. 4.3 (SAS Institute, Cary, NC).

Pathogenicity and fruit infection assays. Virulence for *Cmm* strains (wild-type, Δ*CmEXLX2*, and the complemented mutant) were prepared and adjusted to an OD_{600nm}= 0.8 as described above, and inoculated into 3 week old tomato seedlings by the cotyledon clipping method (n=5/treatment) (Xu et al. 2010). Tomato plants were screened daily for characteristic wilting and chlorosis associated with bacterial canker as previously described (Balaji et al. 2008; Tancos et al. 2015). Disease severity was quantified by counting the number of individual leaflets wilting relative to the total number of individual leaflets present on the three oldest leaves. Observations continued until all plants were wilting or until 21 days post inoculation (DPI). The mean area under the disease progress curve (AUDPC) was calculated from disease severity

(Madden et al. 2007). Significant differences among treatments for AUDPC were tested with PROC GLIMMIX ($P<0.05$) followed by Tukey-Kramer posttest ($P<0.05$) using SAS v. 4.3 (SAS Institute, Cary, NC). Each of the three treatments had five plants per replicate, and the entire experiment was performed three times.

Tomato seedlings (n=5/treatment) were harvested at 21 DPI and extra tomato seedlings (n=3/treatment) were previously harvested at 9 DPI in order to quantify i*n planta Cmm* populations. A 0.5 cm section of tomato stem tissue was harvested 1 cm above the inoculation site and subsequently homogenized in 500 µl of sterile 10 mM $MgCl_2$ via a sterile 5 mm stainless steel grinding bead (Qiagen, Valencia, CA) using a TissueLyser (Retsch, Newtown, PA) at 30 Hz for 6 minutes. After homogenization, 500 µl of sterile 10 mM $MgCl_2$ was added to bring the total volume to 1 ml (Balaji and Smart 2012; Tancos et al. 2013). Subsequent dilutions and population counts were performed as previously described (Tancos et al. 2013). The experiment was performed twice. Data were analyzed using PROC GLIMMIX ($P<0.05$) in SAS v. 4.3 (SAS Institute, Cary, NC) and significant differences among experiments were tested using Tukey-Kramer's honestly significant difference test ($P<0.05$). When no significant differences were found between experiments ($P<0.05$), the experiment factor was dropped from the model, and plants were considered reps.

Immature green tomato fruit (8-12 mm in diameter) were inoculated with *Cmm* wild-type, mutant, and the complemented mutant at a density of 10^8 cells/ml using a #2 horse-hair paintbrush as previously described (Medina-Mora et al. 2001; Tancos et al. 2013). Tomato fruit were also brushed with sterile distilled water as a negative control. Fruit were harvested (n=4/strain) approximately 16 days post inoculation and divided into five or six equal vertical cross-sections. All of the lesions represented within two of the randomly selected sections were

measured using an Olympus SZX2 dissecting microscope (Olympus Corp., Shinjuku, Tokyo, Japan) connected to a Nikon Digital Sight-Qi1Mc camera (Nikon Corp., Chiyoda, Tokyo, Japan). Nikon's NIS-Elements V. 4.1 software (Nikon Corp., Chiyoda, Tokyo, Japan) was used to measure the total lesion diameter, necrotic center diameter, and the width of the lesion's 'white halo', at the widest point of the respective variables, for 100 individual lesions/strain. Significant differences among treatments for mean lesion size were tested with PROC GLIMMIX ($P<0.05$) followed by Tukey-Kramer posttest ($P<0.01$) using SAS v. 4.3 (SAS Institute, Cary, NC). The experiment was performed twice.

Virulence of *Rs* isolates was determined on tomato plants following soil-soaking and cut-petiole inoculations. For *Rs* assays, tomato plants (cv. Bonny Best) were grown in ReadyMix Potting Soil in a 28℃ climate controlled chamber with a 12 hr day/12 hr night cycle. At 14 days post sowing, seedlings were transplanted into individual 5-inch pots. For soil-soaking inoculations, strains were grown overnight in 100 ml CPG in 250 ml flasks. Cultures were washed once in water and resuspended in water to $OD_{600\ nm}$=0.200. Bacterial suspensions (50 ml per plant) were poured into the soil of 17-day old plants with unwounded roots (n=15 plants/strain), which corresponds to an inoculum of ~$5x10^8$ CFU/g soil. For cut-petiole inoculations, strains were grown overnight in 5 ml CPG in test tubes. Cultures were washed once, and cell density was adjusted to ~250 CFU/µl. The oldest petiole of 21-day old plants was delicately removed with a sharp razor blade. A 2 µl drop containing 500 CFU was placed on the cut petiole. For both *Rs* virulence assays, wilting symptoms of each plant were rated daily on a 0-4 disease index scale: 0, no symptoms; 1, ≤ 25% leaves wilted; 2, ≤ 50% leaves wilted; ≤ 75% leaves wilted; ≤ 100% leaves wilted.

***Cmm* movement and its influence on *in planta* growth.** To assess differences for *in planta* movement, *Cmm* wild-type, mutant, and the complemented mutant were prepared and adjusted to an OD_{600nm}= 0.8 as described above. Acropetal movement treatments utilized three week old tomato seedlings (3-true leaf stage) and the cotyledon clipping method (n= 3/treatment). To ensure *Cmm* strains were confined to the vasculature of the stem and not taking alternate vascular routes via the leaves (biasing movement patterns), the first two true leaves were aseptically removed prior to inoculation. Tomato seedlings were harvested at 21 DPI in order to quantify i*n planta* bacterial populations at disparate distances. A 0.5 cm section of tomato stem tissue was harvested 5 cm and 10 cm above the inoculation site and subsequently processed to determine bacterial populations as described above. This was also performed with the negative control plants except sterile water was used for the cotyledon clip. The experiment was performed three times with a total of 9 plants/strain. Data were analyzed using PROC GLIMMIX ($P<0.05$) in SAS v. 4.3 (SAS Institute, Cary, NC) and significant differences among experiments were tested using Tukey-Kramer's honestly significant difference test ($P<0.05$). When no significant differences were found between experiments ($P<0.05$), the experiment factor was dropped from the model, and plants were considered reps.

Assessing differences in the lateral movement of *Cmm* strains. Visualization of *in planta* movement was assessed by transforming of wild-type and Δ*CmEXLX2* strains with the eGFP transient expression vector pK2-22 (Chalupowicz et al. 2012) using the previously described protocol. EGFP-expressing strains were prepared and adjusted to an OD_{600nm}= 0.8 as described above and inoculated into 3 week old tomato seedlings by the cotyledon clipping method. Tomato plants (n=6/treatment/time point) were harvested and screened at 5 DPI, 7 DPI, and 9 DPI. At each respective time point, microscopic analysis was performed on tomato stem cross-

sections taken at 1, 2, and 3-cm above the inoculation site and -1, -2, and -3-cm below the inoculation site. Cross-sections were assessed for the number of protoxylem, vascular bundles, and xylem parenchyma cells infected with the respective eGFP-expressing strains. All plant sections were hand sliced with sterile double edged razors and visualized using an Olympus BX61 microscope connected to a confocal laser scanning microscope (CLSM) system (Olympus Fluoview FV-300, Melville, NY). An argon laser (488 nm excitation) and a green helium neon laser (543 nm excitation) was used to excite the eGFP bacteria and induce plant autofluorescence, respectively (Dunn et al. 2013). Negative control tomato stems were also harvested and analyzed.

Crystal violet staining assay for *Cmm* attachment. Differences in bacterial attachment between Δ*CmEXLX2*, wild-type, and the complemented mutant were assessed with three separate media including: pure tomato xylem sap, xylem mimicking medium (Hiery et al. 2013) or LB medium. Bacterial isolates were grown in LB broth (gentamicin and/or kanamycin were added for Δ*CmEXLX2* or the complemented mutant) as described above for 24 hours, adjusted to OD_{600nm}= 0.8, pelleted at 3041 x g for 6 minutes, washed and centrifuged, and resuspended in sterile water. Bacterial suspensions (125 µl) were added to the individual wells of a 24-well Falcon tissue culture plate (Corning Inc., Corning, NY) containing 375 µl of pure tomato xylem sap, xylem mimicking medium, or LB medium. Within each plate all 24 wells contained 375 µl of the same medium with 18 wells inoculated with the bacterial suspension and the remaining 6 wells inoculated with distilled water as the negative control. The plates were briefly agitated and placed at 27°C for five days without further agitation. The plates were subsequently inverted and poured out, vigorously washed twice by submerging the plates in a water bath, blotted onto paper towels and placed at 60°C for 1 hour (Davey and O'Toole 2000; Kwasny and Opperman 2010).

The adherent bacteria were stained with 500 µl of 0.1% crystal violet for 15 minutes at room temperature, followed by gently washing the plates three times by submerging the plates in distilled water (Davey and O'Toole 2000). Crystal violet was solubilized by adding 500 µl of 30% acetic acid, followed by agitating the plates and quantifying the absorbance at 590 nm using the BioTek micoplate reader (Kwasny and Opperman 2010; Davey and O'Toole 2000). The experiment was performed twice with independently-derived media for a total of 36 absorbance readings/strain/medium. Significant differences among treatments were tested with PROC GLIMMIX ($P<0.05$) followed by Tukey-Kramer posttest ($P<0.05$) using SAS v. 4.3 (SAS Institute, Cary, NC).

Root attachment assays. To assess root attachment with *Cmm* strains, Mountain Fresh Plus tomato seeds were germinated on wet sterile filter paper in 135 mm petri dishes (Fisher Scientific; Pittsburgh, PA) until seedling roots were approximately 2 cm in length. *Cmm* wild-type, mutant, and complemented mutant were grown and adjusted to an OD_{600nm}= 0.6 as previously described. Emergent seedlings were individually collected (n=20/strain), placed on 1% water agar plates, inoculated along the root of each seedling with 10 µl of bacterial suspension, and incubated at room temperature for 2 hours. Following incubation, the roots were aseptically removed and pooled in groups of four for each respective strain, and gently washed twice in 20 ml of 10 mM $MgCl_2$ to remove any non-adherent bacteria. The pooled roots (n=4) were subsequently homogenized in 500 µl of sterile 10 mM $MgCl_2$ via a sterile 5 mm stainless steel grinding bead (Qiagen, Valencia, CA) using a TissueLyser (Retsch, Newtown, PA) at 30 Hz for 2 minute (Balaji and Smart 2012; Tancos et al. 2013). Subsequent dilutions and population counts were performed as previously described (Tancos et al. 2013). The experiment was performed three times. Significant differences among treatments for root attachment were

tested ANOVA ($P<0.05$) followed by Tukey's studentized posttest ($P<0.05$) using SAS v. 4.3 (SAS Institute, Cary, NC).

The same assay was used to evaluate the ability of *Rs* strains to attach to tomato roots. Briefly, tomato seeds (cv. Bonny Best) were surface-sterilized by rinsing for 10 min in 10% bleach followed by 10 min in 70% ethanol. The seeds were washed five times with sterile water and placed on 1% agar plate overlaid with sterilized Whatman N°1 filter paper (GE Health Care, Pittsburg, PA). Axenic seeds were germinated in the dark at 28°C for three days. Seedlings with 2-cm root length were transferred to a new 10 cm square agar plate and incubated with 10 µl of a 10^6 CFU/ml bacterial suspension pipetted along the root axis. Inoculated tomato seedlings were incubated at room temperature for two hours before the roots were excised and washed twice in sterile water to remove non-adherent bacteria. Four roots were pooled together, ground and dilution plated on solid CPG as a technical replicate. The experiment was repeated in three times, each with 10 technical replicates per treatment. Data was analyzed using GraphPad Prism 6.0 (GraphPad Software, La Jolla, CA).

ACKNOWLEDGEMENTS

M. Tancos was supported by grant no. DGE-1144153 from the National Science Foundation-Graduate Research Fellowships Program. Additional support was provided by federal formula funds administered through the New York State Agricultural Experiment Station. T. Lowe-Power was funded by NIH National Research Service Award T32 GM07215 and by Agriculture and Food Research Initiative Competitive Grant # 2015-67011-22799 from the USDA National Institute of Food and Agriculture. T. Tran was supported by NSF Grant # IOS1456636. Special

thanks to Shaun Stice, Chase Crowell, and members of the Smart lab for assisting with various experiments. We would also like to thank Dr. Tom Burr for critical reading of the manuscript.

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CONCLUSIONS

The focus of my dissertation was to better understand the population diversity and *in planta* movement of the Gram-positive phytopathogen *Clavibacter michiganensis* subsp. *michiganensis*. Relatively few studies have focused on the infection processes of Gram-positive bacteria, since the majority of pathogens (animal and plant) are Gram-negative. However, the *C. michiganensis* subsp. *michiganensis*-tomato pathosystem is an ideal system to study, since both pathogen and host are sequenced, and preliminary methods are available for *C. michiganensis* subsp. *michiganensis* transformation and site-specific mutagenesis. Additionally, tomato is a globally important agricultural crop, and *C. michiganensis* subsp. *michiganensis* is an equally important pathogen that continues to inflict significant damage. The knowledge of how *C. michiganensis* subsp. *michiganensis* systemically colonizes tomato will help elucidate mechanisms of pathogenicity, and advance our basic understanding of pathogenic Gram-positive bacteria.

I was fortunate to be awarded an NSF-GRFP fellowship during my doctoral research, which provided the flexibility to pursue new areas of research that I could design and implement. Since little is known about *C. michiganensis* subsp. *michiganensis* pathogenesis, many questions and research topics remained unexplored. When I first started this project, we wanted to better characterize the New York *C. michiganensis* subsp. *michiganensis* population in order to determine if novel haplotypes were entering New York through infected tissue or if haplotypes were persisting on farms. The diversity study revealed the number of unique haplotypes, and the importance of putative virulence genes, plasmid profiles and how virulence genes persisted within natural field populations. In association with the diversity study, speculative evidence

supported the hypothesis of routes of seed infection, but detailed histopathological studies were lacking. Therefore, our objective was to characterize routes of seed infection through possible entry points. Based on this histopathological approach, unique phenotypic attributes were observed during infection, which led to new questions involving xylem movement, cellular infections, and colonization. My final objective was to elucidate the role of putative virulence genes during infection while utilizing some of the recently characterized New York field strains. The roles of these newly characterized genes provided valuable insight into the infection process of *C. michiganensis* subsp. *michiganensis* and its repertoire of virulence-associated genes.

Diversity of the New York *Clavibacter michiganensis* subsp. *michiganensis* population

Based on our preliminary research, the *C. michiganensis* subsp. *michiganensis* New York population appeared to be very diverse with all six known BOX-A1R patterns represented. Unfortunately, the benefits of utilizing repetitive element-PCR as a method of determining population structure, whether within fields or regions, was limited because of its low discriminatory power and the difficulty in identifying genotypes following gel electrophoresis. Therefore, we further characterized our population with a multilocus sequence analysis (MLSA) approach, which comprised five housekeeping genes and three putative virulence genes, providing a discriminatory power of 0.946. The MLSA approach provided higher resolving power than the current repetitive element-PCR approach by differentiating the 51 isolates into 21 unique haplotype groups. This enabled a detailed interpretation of the diversity and potential movement of *C. michiganensis* subsp. *michiganensis* isolates within New York.

Several of the collected isolates were reoccurring strains, with isolates being maintained in or around the surrounding area for several years. The preservation of identical haplotypes

over an extended period highlights the difficulty in properly managing bacterial canker due to misdiagnosis and improper sanitation. Conversely, many isolates were novel and appeared on farms with no previous history of bacterial canker of tomato, suggesting recent introduction events from infected seed or transplants.

The presence or absence of putative virulence genes was also determined in order to better define the New York population. The majority of isolates were pathogenic, but differences in disease incidence were observed. PCR analysis indicated that the presence of putative virulence genes varied between isolates and highlighted the ephemeral nature of virulence genes in field populations. The association of aggressiveness with putative virulence genes and/or plasmids was not always absolute. Several isolates with plasmids and all six putative virulence genes produced symptoms identical to isolates lacking some virulence genes and plasmids. This further demonstrates the variability in virulence observed among a diverse population of field isolates, and the hypothesized importance of putative virulence genes. Similarly, plasmid profiles were unique, diverse, and different from both Serbian and Israeli *C. michiganensis* subsp. *michiganensis* populations.

Besides looking at the genetic diversity at a statewide level, we wanted to better understand global *C. michiganensis* subsp. *michiganensis* populations, and a recent study from Serbia enabled such a comparison. When comparing the New York and Serbian isolates, 98% of the genetic variation occurred within populations and only 2% genetic variation occurred between the two geographically-separated populations, which is indicative of a common origin or movement event. Overall, having the ability of utilizing MLSA to identify strains is a major advance in our ability to identify the source of inoculum and implement appropriate disease management strategies.

Tomato fruit and seed colonization

The stable transformation of *C. michiganensis* subsp. *michiganensis* with constitutive eGFP-expression permitted detailed *in situ* visualization of entry routes necessary for fruit and seed colonization. Systemically, *C. michiganensis* subsp. *michiganensis* could access seed through the xylem, with seed infections becoming more common when the floral units were between the bud and pre-anthesis stages at the time of infection. In contrast to systemic infections, eGFP-labeled *C. michiganensis* subsp. *michiganensis* also proliferated within lesions on the fruit exocarp and subsequently spread into the fruit mesocarp when externally inoculated. Confinement of bacteria to the inter- and intracellular regions of the lesions during the earlier stages of fruit development was quickly lost once fruit began to ripen, suggesting that the weakened cellular components facilitated bacterial spread into the thin-walled mesocarp cells and xylem of the pericarp. Once in the xylem, the pathogen spread through the fruit and entered the plant through the pedicel. Lesion-to-vascular ingression most likely occurred near the distal ends of the fruit where vascular elements were noticeably closer to the epidermis.

These results highlight the ability of *C. michiganensis* subsp. *michiganensis* to invade tomato fruit and seed through multiple entry routes. Irrespective of inoculation method (systemic or external), *C. michiganensis* subsp. *michiganensis* was observed within and around developing tomato seed. The bacteria were located within the developing seed, endosperm, and funiculus, although at relatively low levels compared with the large numbers of bacteria observed in xylem and pericarp cells. The ability of *C. michiganensis* subsp. *michiganensis* to access the seed, in combination with no external fruit or plant symptoms (with systemic infections), highlights the difficulty in identifying potentially contaminated seed sources. This further demonstrates the challenges in detecting and eradicating small initial pathogen

populations within seed lots. Overall, characterizing the routes of seed infection provides insight into the versatile nature of this pathogen and possibly insight into seed infection mechanisms that will aid in necessary control measures.

Contrasting roles of plant-like bacterial expansins with two tomato vascular pathogens

Our research demonstrated contrasting bacterial expansin functions for two distinctly different vascular bacterial pathogens of tomato, *C. michiganensis* subsp. *michiganensis* and *R. solanacearum*, which independently acquired plant-like expansins and selectively modified their functions to have diverse roles in host colonization. Following inoculation with *C. michiganensis* subsp. *michiganensis* strains, Δ*CmEXLX2* induced greater disease severity with the rapid onset of unilateral wilting and numerous atypical necrotic lesions forming on the vasculature of petioles, when compared to the wild-type and complemented mutant. Necrosis appeared to be confined to the vasculature with healthy tissue surrounding the lesions until later stages of disease. Similar to increased symptom development of infected plants, fruit lesions became significantly larger and more necrotic following inoculation with Δ*CmEXLX2*. Fruit with Δ*CmEXLX2* lesions appeared more blistered, larger, more necrotic and with less noticeable 'white halos'. Moreover, *in planta* bacterial populations were significantly different between *C. michiganensis* subsp. *michiganensis* strains with the *CmEXLX2* mutant having larger populations (1 cm above the site of inoculation) at both 9 and 21 DPI. However, population differences between strains were no longer significant as acropetal regions of the stem were colonized.

Knocking out *CmEXLX2* in *C. michiganensis* subsp. *michiganensis*, resulted in increased necrosis and larger *in planta* populations, but the molecular basis by which CmEXLX2 is influencing necrosis remains unknown. The greater rates of necrosis observed on the plant and

fruit may be directly associated with the increased availability of plant-derived nutrients and subsequent influx of bacterial growth. Contrary to *C. michiganensis* subsp. *michiganensis*, the Allen Lab (University of Wisconsin-Madison) demonstrated that the *R. solanacearum* expansin mutant had negligible effects when directly introduced into the tomato vasculature. However when introduced into the soil (a more natural route of infection), the *R. solanacearum* expansin mutant Δ*RsEXLX* resulted in significantly less disease, possibly because the mutant hyper-attached to the roots and was less efficient at root colonization.

Bacterial non-chimeric expansins appear to have undergone intense selective pressures to supplement their host's lifestyle. For *C. michiganensis* subsp. *michiganensis*, CmEXLX2 appears to influence vascular and fruit necrosis leading to larger *in planta* populations and symptom progression within tomato. While RsEXLX appears to influence root internalization and attachment for *R. solanacearum* infections. The conservation of this plant xenolog in vascular-colonizing phytopathogenic bacteria does not imply that the function is xylem limited. Instead increased necrosis appearing on the tomato fruit and the vasculature with *C. michiganensis* subsp. *michiganensis* infections, leads us to speculate that vascular-inhabiting bacterial pathogens horizontally acquired and maintained the expansin gene due to their highly acidic microenvironments.

Future directions

The exciting results generated from the aforementioned chapters led to many new questions, which are currently being designed and implemented. Both the Allen and Smart labs are performing "protein fusion" assays with the non-chimeric bacterial expansins of *C. michiganensis* subsp. *michiganensis* and *R. solanacearum*. In order to determine if expansin

functions are conserved in vascular colonization of tomato, the *R. solanacearum* expansin domains (D1/D2) will be attached to the *C. michiganensis* subsp. *michiganensis* expansin promoter sequences, and vice versa with the expansin domains of *C. michiganensis* subsp. *michiganensis*. The subsequent "fusions" will be introduced into the respective expansin mutant strains to determine if the Gram-negative *R. solanacearum* expansin can restore the wildtype phenotype in the Gram-positive *CmEXLX2* mutant or vice versa. These "fusion" assays will further refine or modify our initial hypotheses regarding the contrasting functions of bacterial non-chimeric expansins. Additional studies could further explore the molecular mechanisms of bacterial expansins, including: expansin localization (GFP-tagged or fluorescently-tagged antibodies), protein interactions, conservation of enzymatic sites (between *C. michiganensis* subsp. *michiganensis* and *R. solanacearum*), binding competition assays, and regulatory differences.

Clavibacter michiganensis would be the ideal Gram-positive phytopathogenic model system for future expansin studies because its host range includes both monocots and dicots. The recent sequencing of the *C. michiganensis* subsp. *tessellarius* genome (causal agent of bacterial mosaic of wheat) reveals a putative non-chimeric expansin, which shares 73% amino acid sequence similarity to *CmEXLX2*. It appears that *C. michiganensis* subsp. *tessellarius* and *C. michiganensis* subsp. *michiganensis* expansins are most similar in amino acid sequence, and appear to be the only non-chimeric expansins present within the *Clavibacter* genus. Because of expansin sequence similarities and host-type differences, exploring the interactions of bacterial expansins to monocotyledonous and dicotyledonous cell walls could further elucidate conserved bacterial expansin motifs and putative binding sites.

The primary cell walls of dicots (type I) and monocots (type II) vary significantly in both structure and architecture. Both cell walls have the same cellulose microfibril matrix, but type I primary cell walls (dicots) utilize xyloglucan as the main hemicellulose with a pectin-rich composition. Conversely, type II walls (monocots) use glucuronoarabinoxylan as the main hemicellulose with an overall pectin-poor matrix and few structural proteins (Cosgrove 2005; Pattathil et al. 2015). In order to modify and loosen primary cell walls, vascular plants evolved divergent expansins represented by α-expansin, β-expansin, expansin-like A, and expansin-like B families (Cosgrove 2015). The α-expansins appear to play a dominant role in dicots, while the β-expansins appear to be more prevalent in grasses and other monocots (Cosgrove 2015; Sampedro et al. 2015). Current research has primarily focused on the properties of α-expansins, in which progress has been made involving putative cell wall binding sites and the importance of biomechanically important xyloglucan-cellulose junctions (Wang et al. 2013; Silveira and Skaf 2016; Sampedro et al. 2015). However, the targets of β-expansins remain unknown (Sampedro et al. 2015). Future studies may determine if the *C. michiganensis* subsp. *tessellarius* and *C. michiganensis* subsp. *michiganensis* expansins are cross-functional in these diverse primary cell walls, thereby allowing the further characterization of bacterial expansins in pathogenesis.

Final thoughts

Overall, many questions remain unanswered regarding the pathogenesis, molecular interactions, and movement of *C. michiganensis* subsp. *michiganensis*. For example, past experiments demonstrated that curing *C. michiganensis* subsp. *michiganensis* of its plasmids (and their associated virulence genes) resulted in the reduction of disease symptoms, with *in planta* populations remaining unchanged (Meletzus et al. 1993). This observation and the

common occurrence of symptomless infections, resulted in the categorization of *C. michiganensis* subsp. *michiganensis* as a biotroph with an initial endophytic vascular phase (Meletzus et al. 1993; Bermpohl et al. 1996; Eichenlaub and Gartemann 2011). However based on my research, I would instead label *C. michiganensis* subsp. *michiganensis* as a "vascular necrotroph".

C. michiganensis subsp. *michiganensis* appears to have a mosaic of different life-styles that distinguish it from true biotrophs and necrotrophs; therefore, *C. michiganensis* subsp. *michiganensis* should be placed in a unique category termed "vascular necrotroph" because it subsists in metabolically-inactive xylem vessels and interacts minimally with metabolically active cells until high *in planta* titers are achieved. True necrotrophs kill host cells before colonizing, but *C. michiganensis* subsp. *michiganensis* initially proliferates in non-living vessels in a saprophytic-like manner. Once large titers are reached, *C. michiganensis* subsp. *michiganensis* appears to switch to a necrotrophic mode of action, which results in the lateral movement and maceration of surrounding metabolically-active parenchyma cells. In addition, *C. michiganensis* subsp. *michiganensis* possesses an arsenal of cell wall-degrading enzymes that rupture primary cell walls and spiral thickenings 4-5 days post inoculation (Wallis 1977). The relatively rapid destruction of tissue, after an initial growth phase, does not support a biotrophic lifestyle and may explain why tomato resistance genes are lacking for this "vascular necrotroph".

Evolutionarily considered, *C. michiganensis* subsp. *michiganensis* is a truly effective bacterial phytopathogen due to its ability to persist in the environment, overcome host defenses, become vertically-transmitted with its host, and ultimately attain global distribution. Regardless of its mode of infection, scientific discoveries continue to unfold the complexity of this pathosystem and provide new insights into Gram-positive phytopathogenesis.

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APPENDIX

INVESTIGATING THE ROLE OF A PUTATIVE β-N-ACETYLGLUCOSAMINIDASE IN *C. MICHIGANENSIS* SUBSP. *MICHIGANENSIS* INFECTIONS

INTRODUCTION

Clavibacter michiganensis subsp. *michiganensis* utilizes numerous secreted cell-wall-degrading-enzymes, serine proteases, and other uncharacterized proteins to successfully colonize the vasculature of tomato (Gartemann et al. 2008; Savidor et al. 2012). Expression data from several *in vitro* and *in planta* studies have demonstrated the up-regulation of numerous genes ranging from ABC transporters to uncharacterized polypeptides, and revealed dozens of potentially important pathogenesis-associated genes (Balaji et al. 2008; Flügel et al. 2012; Savidor et al. 2012; Hiery et al. 2013, 2015). Unfortunately, many of these upregulated genes, and their putative roles in virulence, remain uncharacterized. Within the 129 kb *C. michiganensis* subsp. *michiganensis* pathogenicity island, β-N-acetylglucosaminidase (*nagA*) was the third most upregulated protein in the *C. michiganensis* subsp. *michiganensis* proteome under *in vitro* infection-mimicking conditions (Gartemann et al. 2008; Savidor et al. 2012). The role of a putative β-N-acetylglucosaminidase in *C. michiganensis* subsp. *michiganensis* infections is unknown, but it has been identified as a major virulence factor in the mammalian Gram-positive pathogen *Streptococcus pneumonia* (Bateman et al. 2005; Moscoso et al. 2006; Rico-Lastres et al. 2015). Within *S. pneumonia*, the N-acetylglucosaminidase protein (LytB) is a conserved peptidoglycan hydrolase necessary for bacterial separation, adhesion and invasion of

host cells, biofilm formation, and peptidoglycan modification (Moscoso et al. 2006; Rico-Lastres et al. 2015). Conversely in Gram-negative bacteria, the human pathogen *Salmonella enterica* utilizes a functional β-N-acetylglucosaminidase as an essential flagella subunit, while in the plant pathogen *Xanthomonas campestris* pv. *campestris,* β-N-acetylglucosaminidase modulates virulence through peptidoglycan recycling pathways (Herlihey et al. 2014; Yang et al. 2014). Currently it remains unknown if the *C. michiganensis* subsp. *michiganensis* β-N-acetylglucosaminidase is functional or if it only possesses conserved motifs, but based on recent diversity studies, *nagA* appears to be highly conserved throughout the *C. michiganensis* subsp. *michiganensis* New York population (Tancos et al. 2015). Increased expression in the pathogenicity island, gene conservation within a diverse population, and association with virulence in a variety of bacterial and eukaryotic pathogens, highlights the putative importance of *nagA* in pathogenesis (Qian et al. 2005; Cannon et al. 1994; Kumar et al. 2000; Jermyn and Boyd 2002; Bateman et al. 2005). As a result, the highly upregulated *nagA* gene was selected to be characterized in the *C. michiganensis* subsp. *michiganensis*-tomato pathosystem.

This preliminary study explores the possible role of a putative β-N-acetylglucosaminidase in *C. michiganensis* subsp. *michiganensis* infections of tomato. Our working hypothesis was that the putative β-N-acetylglucosaminidase was beneficial in *C. michiganensis* subsp. *michiganensis* aggregation, vascular colonization, and disease development. The objectives of the study were (i) to explore the role of NagA in bacterial aggregation and adhesion, (ii) to determine the influence of NagA on vascular colonization, and (iii) to investigate disease progression and symptom development in tomato.

MATERIALS & METHODS

All bacterial strains/plasmids and primers used in the construction of *Clavibacter michiganensis* subsp. *michiganensis* (*Cmm*) *nagA* mutants are listed in Table A.1 and A.2.

Table A.1. Bacterial strains and plasmids used in this study

Strain or Plasmid	Description[†]	Reference/Source
Strains		
C. michiganensis subsp. *michiganensis*		
Cmm0767	Wildtype virulent New York strain	(Tancos et al. 2015)
Δ*nagA*	Cmm0767 mutant strain lacking *nagA*	This study
CΔ*nagA*$^{+}$	Δ*nagA* transformed with pHNagA (complement)	This study
Escherichia coli		
Zymo 5α (DH5α)	Cloning strain	Zymo Research
E.coli ER2925	*dam* and *dcm* methylation-negative strain	New England Biolabs
Plasmids		
C. michiganensis subsp. *michiganensis*		
pGEM-T Easy	Cloning vector; Amp^{r}; 3 kb	Promega
pHN216	*E. coli–Clavibacter* shuttle vector; Gm^{r} Nm^{r}; 13.8 kb	(Laine et al. 1996)
pGCMNa	pGEM-T Easy:*nagA*; 4.6 kb	This study
pGCMNaGM-α	pGEM-T Easy:*nagA*::Gm^{r} cassette; Amp^{r} Gm^{r}; 5.3 kb	This study
pHNagA	Full length *nagA* cloned into pHN216; Nm^{r}; 14.2 kb	This study

[†]Gm^{r}, Gentamicin acetyltransferase; Nm^{r}, aminoglycoside (neomycin) phosphotransferase; Amp^{r}, β-lactamase

Table A.2. Oligonucleotides used in this study

Name	Sequence (5'-3')[†]	Target	Purpose	Locus Tag	Tm (°C)	Amplicon size (bp)	Reference
NagA	F:AGAACCTGACAACGGTAGCT R:CAGATTGCGCGTACACACTA	*nagA*	Cloning	CMM_0049	60	1522	This study
GnR-RsrII	F:ACAGCGCGGTCCGACCCAGTTGACATAAGCCTGTTCG R:ACAGCGCGGACCGCGGCTTGAACGAATTGTTAGGTGG	Gentamicin acetyltransferase	Cloning	NA	65	799	This study
Gnx	F:CAGTTGACATAAGCCTGTTCG R:GCTTGAACGAATTGTTAGGTGG	Gentamicin acetyltransferase	Verification	NA	58	768	This study
Eco-upB-a Eco-dnC-a	F: ACAGCGGAATTCTAGCGTCCTCGATACCCATC R: ACAGCGGAATTC TCGCGCTGAGTGCTGACTC	*nagA* upstream *nagA* downstream	Cloning Cloning	CMM_0049 CMM_0049	67	2037	This study
Multiple cloning site	T7:TAATACGACTCACTATAGGG SP6:ATTTAGGTGACACTATAG	pGEM-T Easy	Sequencing	NA	50	NA	
nagdn-ppaB2-a	R:GAATGACCACCTTCGCGTAC	downstream of *nagA* insertion	Sequencing	CMM_0050	60	NA	This study
nagA-upA	F:CTCGTCCGGACCCTCATCAG	Upstream of *nagA* insertion	Sequencing	CMM_0049	60	NA	This study
HI-GC-Insert	F:ATAGGGGTTCCGCGCACATTTCC R:CATCGGAGCGGGGTTCATGTGGCT	*nagA* insert in pHNagA	sequencing	NA	67	2250	This study
GyrB	F:ACCGTCGAGTTCGACTACGA R:CCTCGGTGTTGCCSARCTT	DNA gyrase, subunit B	RNA expression	CMM_0006	60	525	(Zaluga et al. 2013; Richert et al. 2005)

[†]Underline indicates restriction enzyme cut site

Bacterial strains and growth conditions. Bacterial strains and plasmids used in this study are listed in Table A.1. The *Cmm* strain (Cmm0767) used in the present study was a virulent New York field strain (Tancos et al. 2015). Depending on the assay, *Cmm* isolates were incubated for 3-4 days at 27°C in Luria-Bertani (LB) (Miller 1972), SB (Kirchner et al. 2001; Stork et al. 2008), or D2ANX media (Hadas et al. 2005). When required, LB medium was supplemented with the antibiotics gentamicin (40 µg/ml), kanamycin (100 µg/ml), or ampicillin (100 µg/ml) (Fisher Scientific; Pittsburgh, PA). All *Cmm* isolates were stored in 35% glycerol at -80°C.

Construction of plasmids pGCMNaGM-α and pHNagA. The *Cmm* putative β-N-acetylglucosaminidase gene, *nagA*, was amplified with primers NagA-F/R (Table A.2) to generate a 1522-bp PCR product, which was cloned into a pGEM-T Easy vector (Promega Corp, Madison, WI) yielding pGCMNa. A gentamicin resistance cassette (produced from pHN216 (Laine et al. 1996)) with artificial *Rsr*II restriction sites was PCR amplified, and subsequently ligated into the single *Rsr*II site of *nagA* in pGCMNa to yield pGCMNaGM-α using *E.coli* Zymo 5α (Zymo Research Inc., Irvine, CA). The pGCMNaGM-α was subsequently transferred into *dam*$^-$ and *dcm*$^-$ *E.coli* ER2925 (New England Biolabs, Ipswich, MA) in order to increase transformation efficiency in *Cmm* (Table 3.5) (Kirchner et al. 2001). For complementation studies, the native promoter and full-length *nagA* gene (based on *Cmm* strain NCPPB382) with artificial *Eco*R1 cut sites was synthesized with Eco-upB-a and Eco-dnC-a primers (Table A.2). The full-length *nagA*-*Eco*RI product was digested using *Eco*R1 and cloned into compatible sites within pHN216 (Laine et al. 1996), replacing the aminoglycoside (neomycin) phosphotransferase gene and producing pHNagA.

Transformation and isolation of mutants. DNA of pGCMNaGM-α and pHNagA was extracted from *dam*$^-$ and *dcm*$^-$ *E.coli* ER2925 with E.Z.N.A Fastfilter plasmid midi kit (Omega

Bio-Tek; Norcross, GA). Competent cells were prepared and transformed as previously described by Stork et al. (2008) and Tancos et al. (2013), respectively. The vector pGCMNaGM-α was electroporated into the wildtype *Cmm* strain 0767 (Cmm0767) yielding the *nagA* mutant (Δ*nagA*). Sequencing of upstream and downstream regions of the insert in the mutant strain Δ*nagA* confirmed that the cassette was correctly inserted. The wildtype *nagA* expressing vector, pHNagA, was subsequently electroporated into the mutant strain Δ*nagA*, yielding the complemented mutant CΔ*nagA*$^{+}$. Putative *Cmm* mutants were grown on LB medium amended with gentamicin (40 µg/ml) and/or kanamycin (100 µg/ml) and selected following 3-4 days growth at 27°C.

PCR and sequencing analysis. Confirmation of plasmid constructs and chromosome integrations were confirmed with gene-specific PCR and sequencing (Table A.2). Genomic *Cmm* DNA was extracted with the MasterPure Gram-Positive DNA purification kit (Epicentre, Madison, WI) according to the manufacturer's instructions. Amplification was performed in a C1000 Touch Thermal Cycler (Bio-Rad Laboratories, Inc., Hercules, CA) using EmeraldAmp GT PCR Master Mix (Takara Bio Inc., Otsu, Shiga, Japan) according to the manufacturer's instructions. Extracted total DNA was used at approximately 50 ng per reaction with 10 µM each of forward and reverse primers (Table A.2). PCR was performed in 25 µl reactions with the following parameters: initial preheat for 3 min at 95°C, 35 cycles at 95°C for 15 sec, a primer pair specific annealing temperature for 30 sec (Table A.2), 72°C for 2 min, a final extension at 72°C for 5 min, and held at 12°C. PCR products were purified with a DNA Clean & Concentrator – 25 kit (Zymo Research Inc., Irvine, CA) according to the manufacturer's instructions. The cleaned PCR amplicons were sequenced on a 3730XL DNA Analyzer (Applied Biosystems) at the Cornell University Life Sciences Core Laboratories Center.

Crystal violet staining assay for *Cmm* attachment. Differences in bacterial attachment between Δ*nagA*, wildtype, and the complemented mutant were assessed with pure tomato xylem sap. Bacterial isolates were grown for 28 hours in LB broth (gentamicin and/or kanamycin were added for Δ*nagA* or the complemented mutant), adjusted to OD_{600nm}= 0.8, pelleted at 3041 x g for 6 minutes at 4°C, washed and centrifuged, and resuspended in sterile water. Bacterial suspensions (125 µl) were added to the individual wells of a 24-well Falcon tissue culture plate (Corning Inc., Corning, NY) containing 375 µl of pure tomato xylem sap. Within each plate all 24 wells contained 375 µl of tomato sap with 18 wells inoculated with the bacterial suspensions and the remaining 6 wells inoculated with distilled water as the negative control. The plates were briefly agitated and placed at 27°C for five days without further agitation. The plates were subsequently inverted and poured out, vigorously washed twice by submerging the plates in a water bath, blotted onto paper towels and placed at 60°C for 1 hour (Davey and O'Toole 2000; Kwasny and Opperman 2010). The adherent bacteria were stained with 500 µl of 0.1% crystal violet for 15 minutes at room temperature, followed by gently washing the plates three times by submerging the plates in distilled water (Davey and O'Toole 2000). Crystal violet was solubilized by adding 500 µl of 30% acetic acid, followed by agitating the plates and quantifying the absorbance at 590 nm using the BioTek micoplate reader (Kwasny and Opperman 2010; Davey and O'Toole 2000). The experiment was performed three times for a total of 18 absorbance readings/strain (n=6). Significant differences among treatments were tested with PROC GLIMMIX ($P<0.05$) followed by Tukey-Kramer posttest ($P<0.05$) using SAS v. 4.3 (SAS Institute, Cary, NC).

RESULTS & DISCUSSION

β-N-acetylglucosaminidase is the third most upregulated protein under infection-mimicking conditions in the *C. michiganensis* subsp. *michiganensis* proteome, but its role in pathogenesis remains unknown (Savidor et al. 2012). The N-acetylglucosamine metabolic family has been associated with virulence in a variety of bacterial and eukaryotic pathogens (Qian et al. 2005; Cannon et al. 1994; Kumar et al. 2000; Jermyn and Boyd 2002; Bateman et al. 2005; Rico-Lastres et al. 2015). Within the mammalian pathogen *Streptococcus pneumonia,* β-N-acetylglucosaminidase is hypothesized to influence bacterial attachment (Bateman et al. 2005). In order to elucidate the role of *nagA* in *C. michiganensis* subsp. *michiganensis,* the native *nagA* gene from a virulent *C. michiganensis* subsp. *michiganensis* New York field strain (Cmm0767) was replaced with a *nagA*::Gm^r cassette without downstream frameshift mutations. Because of its potential role in attachment and colonization, preliminary *in vitro* crystal violet attachment assays were performed with the *Cmm nagA* mutants. All three *Cmm* strains were capable of attachment in tomato sap, but the Δ*nagA* mutant was significantly reduced in its ability to form biofilm-like structures (P=0.0012) in comparison to the wildtype and complemented mutant (Figure A.1). In order to determine if bacterial attachment phenotypes observed *in vitro* for the Δ*nagA* mutant correlate to differences in virulence, 2-3 week old tomato seedlings will be inoculated.

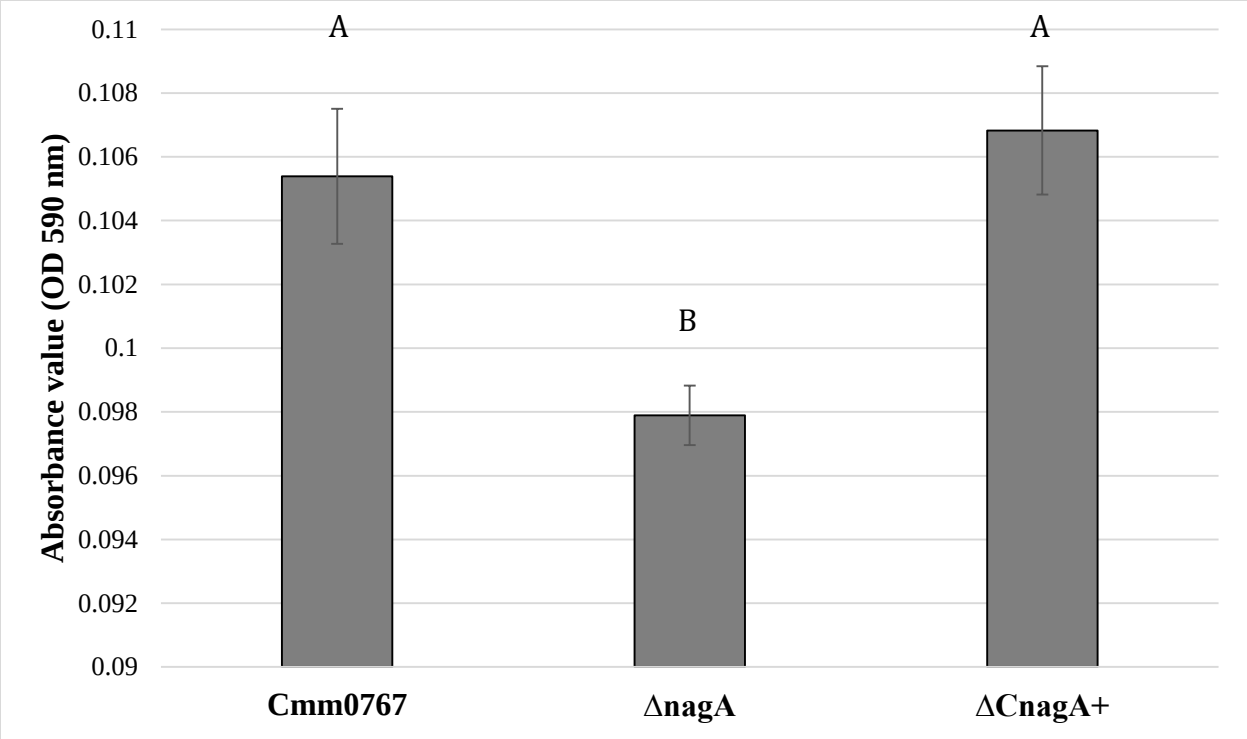

Figure A.1. *In vitro* attachment of *Clavibacter michiganensis* subsp. *michiganensis* Cmm0767 (wildtype), Δ*nagA* (mutant), and CΔ*nagA*$^{+}$ (complement) in pure tomato sap medium. Significant differences among treatments were tested with PROC GLIMMIX ($P<0.05$) followed by Tukey-Kramer posttest ($P<0.05$). Bacterial attachment values followed by the same letter are not significantly different.

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