Characterizing the Genetic Diversity of the *Clavibacter michiganensis* subsp. *michiganensis* Population in New York

Matthew A. Tancos, Holly W. Lange, and Christine D. Smart

Department of Plant Pathology and Plant-Microbe Biology, Cornell University, Geneva, NY.
Accepted for publication 2 September 2014.

ABSTRACT

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.

New York *Clavibacter michiganensis* subsp. *michiganensis* isolates, collected from disparate bacterial canker of tomato outbreaks over the past 11 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*, and *gyrB*) and three putative pathogenicity genes (*celA*, *tomA*, and *nagA*). Repetitive polymerase chain reaction (PCR), with the BOX-A1R primer, confirmed the high diversity of *C. michiganensis* subsp. *michiganensis* isolates in New

York by demonstrating that all six PCR patterns (A, B, 13C, 65C, 81C, and 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 pathogenicity genes varied between isolates and highlighted the ephemeral nature of pathogenicity 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 were not significantly different, with 98% genetic variation within each population and only 2% genetic variation between populations.

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 (23). This gram-positive bacterium is present in many tomato-producing regions, and can rapidly spread globally through contaminated seed (23,43). *C. michiganensis* subsp. *michiganensis* spreads systemically throughout the host's vascular system via the xylem, and radiates outward into the surrounding tissue when high titers are reached (9,43). Once introduced, *C. michiganensis* subsp. *michiganensis* spreads epiphytically by splashing water or mechanically via workers or equipment (5,7). Bacterial canker of tomato can be difficult to control due to the lack of resistant cultivars, ineffective chemical controls, and poor sanitation practices (38,45).

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 and pCM2) (1,12,13). The PAI is approximately 129 kb with a low GC content (64 to 66%), relative to the genome (72.7%), and is subdivided into two regions termed the *chp* and *tomA* subregions (13,26,40). The conjugative plasmids pCM1 (27 kb) and pCM2 (70 kb) can be transferred throughout a population (8). 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, or host-defense suppression (8,12,40).

The majority of *C. michiganensis* subsp. *michiganensis* studies have predominately focused on the pathogenic nature of the

sequenced strain NCPPB382 (9,13,26); however, studies have recently begun to look at the diversity of field isolates, with differences such as plasmid number and presence or absence of putative pathogenicity genes being readily observed with highly virulent field isolates (14,18,28,29,33,47). 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 (47).

DNA fingerprinting techniques such as 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 (17,18,22,24,29,33). 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, and D), and the C PCR pattern can be further segregated into three subgroups (13C, 65C, and 81C) (24). However, the limited differentiation of isolates, incorrect identification of PCR patterns based on the subjectiveness of gels, and difficulty in interlab reproducibility reduces the overall effectiveness of this technique. Conversely, sequencing approaches such as multilocus sequence analysis (MLSA), multilocus sequence typing (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 (20,28,48).

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 11-year period, with an MLSA scheme and (i) identify the diversity among isolates and (ii) compare the New York isolates with other *C. michiganensis* subsp. *michiganensis* populations.

Corresponding author: C. D. Smart; E-mail address: cds14@cornell.edu

<http://dx.doi.org/10.1094/PHYTO-06-14-0178-R>
© 2015 The American Phytopathological Society

MATERIALS AND METHODS

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 semiselective media (Table 1; Fig. 1) (11). Isolate 10-4R was isolated from irrigation water in eastern New York (16). All *C. michiganensis* subsp. *michiganensis* isolates were stored in 35% glycerol at -80°C .

Pathogenicity assays. Tomato (*S. lycopersicum*) 'Mountain Fresh Plus' seedlings were grown in a Cornell potting mix (composed of peat, perlite, and vermiculite in a 4:1:1 ratio) with a photoperiod of 14 h of light and 10 h of darkness in the greenhouse. *C. michiganensis* subsp. *michiganensis* field isolates were grown for 72 h on D2ANX agar plates and inoculated into approximately 3week-old tomato seedlings ($n = 3$ per 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 (36). 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 postinoculation (dpi). Three tomato plants composed a replicate, and the entire experiment was repeated for a total of six 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 h in 30 ml of liquid Luria-Bertani (LB), before being transferred to 500 ml of liquid LB for an additional 24 h. Plasmid DNA was isolated with a Qiagen Large-construct kit (Qiagen, Enlo, The Netherlands) according to the manufacturer's instructions, except that lysozyme at 5 mg/ml was added to the resuspension buffer (reagent P1). All DNA was quantified with a Nanodrop ND-1000 (Wilmington, DE). Plasmid

TABLE 1. *Clavibacter michiganensis* subsp. *michiganensis* isolates used in this study

Isolate	New York county of origin	County symbol	Year collected	BOX-AIR 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	43
13109	Suffolk	SUF	2013	13C	This study
13115	Suffolk	SUF	2013	13C	This study
NCPBP382	United Kingdom	UK	1956	13C	NCPBP
CMM100	United Kingdom	UK	1956	13C	27
04100	Schoharie	SCH	2004	65C	This study
04101	Schoharie	SCH	2004	65C	2
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	16
11015	Albany	ALB	2011	65C	43
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	43
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

diversity was characterized using CHEF-DR II Pulsed-Field Electrophoresis System (Bio-Rad Laboratories, Inc., Hercules, CA) using 6 V/cm, 1% molecular biology-grade agarose, 0.5× Tris-borate-EDTA, and a 0.5- to 10-s linear ramp factor for a run time of 16 h at 14°C. Plasmid DNA was used at 200 ng per well and visualized with ethidium bromide.

Genomic fingerprinting using BOX-A1R rep-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. A fingerprint was generated for each isolate collected using the BOX-A1R primer following a previously described protocol (24,25), 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°C for 1 min, and 65°C for 8 min; a final extension at 65°C for 15 min; and held at 10°C (25). The PCR products (8 μl) were run in a 1.5% agarose gel using 0.5× Tris-acetate-EDTA buffer at 67 V for 18 h at 4°C.

MLSA of *C. michiganensis* subsp. *michiganensis*. Primers for the putative pathogenicity genes—*celA*, *nagA*, *chpE*, *pat-1*, and

phpA—were designed using Primer3 v. 4.0.0 (19,44) and the sequenced *C. michiganensis* subsp. *michiganensis* NCPPB382 genome (Table 2). Primers targeting the housekeeping genes—*kdpA*, *sdhA*, *dnaA*, *ligA*, and *gyrB*—and one additional putative pathogenicity gene (*tomA*) had been previously described (28, 35,48). In order to enhance the discriminatory power of the initial MLST scheme published by Milijašević-Marčić et al. (2012), additional genes were sequenced, including putative pathogenicity genes (48). Multivirulence-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 (10,34,39,49).

The 11 housekeeping and putative pathogenicity genes were analyzed for each isolate using total DNA (as described above) and gene-specific PCR (Table 2). Amplification was performed in a C1000 Touch Thermal Cycler (Bio-Rad Laboratories, Inc.) 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 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 s, a primer-pair-specific temperature for 30 s (Table 2), and 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

Fig. 1. Map of New York with gray-colored counties representing locations where isolates of *Clavibacter michiganensis* subsp. *michiganensis* were isolated. The number of isolates collected from each county is provided in parentheses ($n = 51$).

at 90 V for 45 min, followed by staining with ethidium bromide. All negative PCR reactions were repeated at least twice.

PCR products were purified with a DNA Clean and 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, consisting of five housekeeping genes (*kdpA*, *sdhA*, *dnaA*, *ligA*, and *gyrB*) and three putative pathogenicity genes (*celA*, *tomA*, *nagA*) (Table 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) and manually edited according to peak quality. The sequences were then aligned with Muscle in MEGA 5.2 (42) on an individual gene basis or concatenated. Genes that were missing in a given isolate were treated as deletions in further analyses (46). Consensus sequences were used to generate maximum-likelihood (ML) trees based on the Tamura-Nei model (41) of MEGA 5.2 using all sites, with bootstrapping repetitions of 1,000. Sequences from reference strain *C. michiganensis* subsp. *sepedonicus* NCPPB2137 were used as an out-group (4). Haplotypes were considered unique if the sequence differed by a single nucleotide.

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 (30,31). The concatenated sequences—*ligA*, *kdpA*, *sdhA*, and *tomA*—from seven Serbian *C. michiganensis* subsp. *michiganensis* isolates, representing each of the seven MLST groups, were compared with the same concatenated sequences from each of the 16 housekeeping gene haplotypes observed with the New York *C. michiganensis* subsp. *michiganensis* isolates (28). Only these four genes could be compared because they were used in both studies.

RESULTS

Pathogenicity of New York *C. michiganensis* subsp. *michiganensis* isolates. In total, 51 *C. michiganensis* subsp. *michiganensis* isolates were collected over 11 years (Table 1). Disease

incidence in greenhouse-inoculated plants was consistent, except for six isolates that were only capable of producing disease symptoms in $\leq$ four of the six tomato seedlings tested (Table 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 7 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 from each BOX-A1R pattern (Table 3). NCPPB382 and CMM100 were used as positive and negative controls, respectively. In total, 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 ($n = 2$), and P16 ($n = 2$)—were observed in the New York population of *C. michiganensis* subsp. *michiganensis* (Table 3; Fig. 2). The majority of New York *C. michiganensis* subsp. *michiganensis* isolate plasmid profiles were different from reference strain NCPPB382 (plasmid pattern P1).

BOX-A1R fingerprints. All six BOX-A1R patterns—A ($n = 10$), B ($n = 1$), 13C ($n = 8$), 65C ($n = 16$), 81C ($n = 13$), and D ($n = 3$)—were observed in the New York population of *C. michiganensis* subsp. *michiganensis* within the past 11 years (Tables 1 and 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). 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*, and *gyrB*) and six putative pathogenicity genes (*pat-1*, *celA*, *phpA*, *chpE*, *tomA*, and *nagA*) (Table 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

TABLE 2. Primers used for multilocus sequence analysis

Gene	Sequence (5'-3')	Gene product	Locus tag	Tm (°C)	Amplicon size (bp)	Aligned fragment (bp)	Reference
<i>KdpA</i>	F:GTGCAGAACTTCGTCTCGG R:GAGCATCATGTTGATCATCG	K ⁺ -dependent ATPase	CMM_2751	60	693	670	28
<i>SdhA</i>	F:CCTGGATGTTCTCGTGATCC R:GAGGACATGGAGTTCTTCC	Succinate dehydrogenase	CMM_0970	58	778	749	28
<i>DnaA</i>	F:TACGGCTTCGACACCTTCG R:CGGTGATCTTCTGTTGGCG	Replication initiation factor	CMM_0001	60	933	833	37,48
<i>LigA</i>	F:GTTTCGACGAGCTGAATGC R:CTCGACCTTCTCCATGAC	DNA ligase	CMM_1404	56	544	524	28
<i>GyrB</i>	F:ACCGTCGAGTTCGACTACGA R:CCTCGGTGTTGCCARCTT	DNA gyrase, subunit B	CMM_0006	60	525	501	35,48
<i>CelA</i>	F:GTAGGGACGCAATTCAGAG R:CAATGTCCTTCTTCGCCAGG	Cellulase	pCM1_0020	58	1,240	1,126	This study
<i>TomA</i>	F:CGAACTCGACAGGTTCTCG R:GGTCTCACGATCGGATCC	Tomatinase	CMM_0090	60	529	509	18
<i>NagA</i>	F:AGAACCTGACAACGGTAGCT R:GCTTCGGTTGTTACGGGAA	β -N-acetylglucosaminidase	CMM_0049	60	796	769	This study
<i>ChpE</i>	F:CCTGACGCTTCTACTTCCGA R:ACGTTTGTGCTGGTCCATTC	Serine protease	CMM_0039	58	695	674	This study
<i>Pat-1</i>	F:TGTAGACCGTATAGCCCGTG R:CCTGAGACCTATTACCGCCC	Serine protease	pCM2_0054	55	850	791	This study
<i>PhpA</i>	F:TCTCGCGAATCAGCCCATAT R:CTCCTCTAGTCTTCACGCCC	Serine protease	pCM2_0053	58	709	688	This study

each *C. michiganensis* subsp. *michiganensis* isolate (Table 3). The final MLSA scheme consisted of eight genes consisting of five housekeeping genes (*kdpA*, *sdhA*, *dnaA*, *ligA*, and *gyrB*) and three putative pathogenicity genes (*celA*, *tomA*, and *nagA*) (Table 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 pathogenicity genes were concatenated (Table

TABLE 3. Genetic diversity observed among New York *Clavibacter michiganensis* subsp. *michiganensis* isolates^a

Pattern, isolate ^b	Plasmid			Chromosome					PAI ^c			Profile ^d	DI ^e	Hap ^f
	<i>pat-1</i>	<i>celA</i>	<i>phpA</i>	<i>dnaA</i>	<i>sdhA</i>	<i>gyrB</i>	<i>ligA</i>	<i>kdpA</i>	<i>chpE</i>	<i>tomA</i>	<i>nagA</i>			
13C														
NCPB382	+	+	+	+	+	+	+	+	+	+	+	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
81C														
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
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
A														
0312	+	+	–	+	+	+	+	+	+	+	+	*	6/6	H2
0315	+	+	–	+	+	+	+	+	+	+	+	*	6/6	H2
0314	+	+	–	+	+	+	+	+	+	+	+	P2	6/6	H2
04108	+	+	–	+	+	+	+	+	+	+	+	*	6/6	H7
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: + = gene present, – = gene absent, * = plasmid isolation was not performed, and ** = CMM100 was not included in sequencing analyses because it is identical to *Clavibacter michiganensis* subsp. *michiganensis* strain NCPBP382, minus the two plasmids. Gene location based upon the reference *Clavibacter michiganensis* subsp. *michiganensis* strain NCPBP382.

^b BOX-A1R pattern and isolate.

^c PAI = pathogenicity island.

^d Unique plasmid profile patterns for *Clavibacter michiganensis* subsp. *michiganensis* isolates determined with pulsed-field gel electrophoresis.

^e Disease incidence (DI) expressed as the number of wilting plants per number of inoculated plants 28 days postinoculation.

^f Hap = haplotype: based on the concatenated housekeeping and putative pathogenicity genes.

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, and 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 five haplotypes, 13C into five haplotypes, 65C into nine haplotypes, 81C into four haplotypes, and D into two haplotypes (Table 3). The isolate with the B BOX-A1R pattern (0581) was a member of the H4 haplotype, which included eight 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 (Figs. 3 and 4). Different clustering patterns within the phylogenetic trees were observed when only the housekeeping genes were concatenated (Fig. 3) versus the concatenating of both housekeeping genes and putative pathogenicity genes (Fig. 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 at 2 polymorphic sites (Table 4). Housekeeping genes *kdpA* and *sdhA* showed the greatest amount of variation, with 3.73 and 4.27% of sites, respectively, being polymorphic in each gene, (Table 4). The three remaining housekeeping genes—*dnaA*, *ligA*, and *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 4). Putative pathogenicity-related genes showed minimal sequence variation compared with the housekeeping genes of *C. michiganensis* subsp. *michiganensis*, except for *celA* and *tomA*, which had 1.33 and 0.786% of sites, respectively, being polymorphic in each gene (Table 4).

Polymorphic sites were predominately located within coding sequences but three polymorphic sites (including an indel) were identified in the noncoding sequences of *celA* (Table 4). An identical 20-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 New York, central New York, the capital district, and eastern New York, from 2004 to 2013 (Fig. 1). 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 (Figs. 3 and 4). The indel was treated as a single polymorphic site.

All MLSA gene sequences were deposited in GenBank (accession numbers *dnaA* [KJ723713 to KJ723763], *gyrB* [KJ723764 to KJ723814], *kdpA* [KJ723815 to KJ723865], *ligA* [KJ723866 to KJ723916], *sdhA* [KJ723917 to KJ723967], *tomA* [KJ723968 to KJ724018], *nagA* [KJ724019 to KJ724068], and *celA* [KJ724069 to KJ724116]).

Comparing the genetic diversity of *C. michiganensis* subsp. *michiganensis* isolates from New York and Serbia. In all, 23 concatenated sequences—*ligA*, *kdpA*, *sdhA*, and *tomA*—were aligned, including 7 Serbian and 16 New York *C. michiganensis* subsp. *michiganensis* isolates, each representing a unique haplotype (Table 5). Compared with the reference strain NCPPB382, Serbian isolate P520 had the most polymorphic sites, with 44 differences, and Serbian isolate P137 had the fewest polymorphic sites, with only 11 (Table 5). An AMOVA demonstrated that the two populations were not significantly different ($P = 0.276$), with

Fig. 2. Pulsed-field gel electrophoresis plasmid profiles representing the most diverse plasmid patterns observed in the New York *Clavibacter michiganensis* subsp. *michiganensis* population. Lane 1 = P14 (0317), lane 2 = P16 (0651), lane 3 = P2 (06124), lane 4 = P1 (NCPB382), lane 5 = P15 (0676), lane 6 = P3 (08224), lane 7 = P4 (12083), lane 8 = P10 (13134), lane 9 = P6 (12069), lane 10 = P5 (13048), lane 11 = P7 (04106), lane 12 = P8 (CMM100), lane 13 = P9 (0779), lane 14 = P11 (0572), lane 15 = P12 (0780), lane 16 = P13 (0767).

TABLE 4. Sequence variation in *Clavibacter michiganensis* subsp. *michiganensis* isolates

Gene	Sequenced fragment (bp)	Number of isolates sequenced ^a	Number of haplotypes	Indel ^b	Number of polymorphic sites (%)	Number of polymorphic sites in noncoding sequences	Discriminatory power
Housekeeping							
<i>KdpA</i>	670	52	12	0	25 (3.73)	0	0.868
<i>SdhA</i>	749	52	13	0	32 (4.27)	0	0.878
<i>DnaA</i>	833	52	4	0	8 (0.960)	0	0.597
<i>LigA</i>	524	52	5	0	4 (0.763)	0	0.283
<i>GyrB</i>	501	52	4	0	3 (0.599)	0	0.489
<i>Conc.KdpA-SdhA</i>	1,419	52	17	...	57 (4.02)	...	0.934
<i>Conc.KdpA-GyrB</i>	3,277	52	17	...	72 (2.20)	...	0.934
Pathogenicity							
<i>CelA</i>	1,126	52	8	1	15 (1.33)	3	0.806
<i>TomA</i>	509	52	5	0	4 (0.786)	0	0.630
<i>NagA</i>	769	52	3	0	2 (0.260)	0	0.185
<i>ChpE</i>	674	13	1	0	0	0	0
<i>Pat-1</i>	791	15	1	0	0	0	0
<i>PhpA</i>	688	4	1	0	0	0	0
<i>Conc.</i>							
<i>CelA-NagA</i>	2,404	52	18	...	21 (0.874)	...	0.905
<i>Conc. KdpA-GyrB + CelA-NagA</i>	5,681	52	22	...	93 (1.64)	...	0.946

^a CMM100 was not included in sequencing analyses because it is identical to *Clavibacter michiganensis* subsp. *michiganensis* strain NCPB382, minus the two plasmids.

^b Number of unique indels detected within sequenced fragment. Each indel was treated as a single polymorphic site.

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 an ML phylogenetic tree with no distinct population clusters (Fig. 5). Several New York and Serbian isolates demonstrated high levels of similarity, most notably 0317 and P121.

DISCUSSION

New York *C. michiganensis* subsp. *michiganensis* isolates collected from diseased plants over an 11-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 pathogenicity genes or plasmids was not always absolute. Several isolates with plasmids and all six putative pathogenicity genes produced symptoms identical to isolates lacking some pathogenicity genes and plasmids. Similarly, plasmid profiles were unique, diverse, and different from both Serbian and Israeli *C. michiganensis* subsp. *michiganensis* populations (18,28).

All six BOX-A1R patterns were present in New York, with the C (13C, 65C, and 81C) and A types being the most prevalent, 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 (33). In contrast, 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 (17,18). Unfortunately, the benefits of utilizing rep-PCR as a method of determining population structure, whether within fields or regions, is limited because of its low discriminatory power, subjectiveness, and low interlab 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 pathogenicity 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* (28). 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 (33). Unfortunately, *pat-1* and *celA* were the only shared genes between the current study and the Michigan survey; therefore, population diversity comparisons could not be made (33).

Only one isolate in this study, 09085, lacked *chpE* or *nagA* and was also greatly reduced in virulence. Although we obviously cannot conclude that the losses of these two genes are responsible for reduction in virulence, the potential role of the gene products

Fig. 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 (42).

TABLE 5. Sequence variation in concatenated genes (*ligA*, *kdpA*, *sdhA*, and *tomA*) for *Clavibacter michiganensis* subsp. *michiganensis* isolates from New York and Serbia, in respect to reference strain NCPPB382

Origin, isolate ^a	Sites ^b	SNPs ^c
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 multilocus sequence typing groups (*n* = 7) were selected (28).

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

^c Average number of single-nucleotide proteins (SNPs) (± standard deviation).

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 (36). 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 pneumoniae*, and *Vibrio cholerae* (3,6,15,21,32). Within *V. 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* (15). The functions of these N-acetylglucosamine catabolic genes, especially β -N-acetylglucosaminidase, remain speculative; however, in *S. pneumoniae*, they are hypothesized to provide an adhesive function in biofilm formations (3).

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 (36). Three of the *chp* genes—*chpA*, *chpB*, and *chpD*—are pseudogenes and, although the function of the remaining four

Chp peptides is unknown, they are hypothesized to facilitate plant colonization and nutrient acquisition (40).

Out of the three plasmid-associated pathogenicity-related genes (*phpA*, *celA*, and *pat-I*), 14 isolates lacked at least one of the three, with *phpA* being the most common gene to be lost ($n = 13$) and *celA* 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 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 pathogenicity genes. Similar observations were reported in a study by Yasuhara-Bell et al. (47), who found that field isolates remained pathogenic even though the putative pathogenicity genes *chpC* or *papA* were absent.

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

Fig. 4. Maximum-likelihood phylogenetic tree for the concatenated housekeeping and putative pathogenicity 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 (42).

the greenhouse pathogenicity assays. The presence of this insertion in only six isolates observed over a 10-year period (2004 to 2013) in disparate regions of New York is intriguing but highlights the dynamic nature of pathogenicity genes within populations of *C. michiganensis* subsp. *michiganensis*. Pathogenicity genes within conjugative plasmids, such as is 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 (8).

The ephemeral nature of virulence within field populations of *C. michiganensis* subsp. *michiganensis* can be difficult to study because the isolation of avirulent isolates from diseased plants is relatively rare (12). In our study, however, several isolates (isolated from the same farm over a 7-year period) had differences in putative pathogenicity 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 to 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) (16). 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 pathogenicity-related genes with the same plasmid profiles; however, five SNPs were detected within the *celA* gene, including a 20-bp insertion upstream of the promoter in 0784, thereby differentiating 0690 and 0784. In contrast, 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 4 years later); isolates 0317 and 13048 (identical haplotype based on housekeeping genes but isolated from farms in adjoining counties 11 years apart); and isolates 0310, 0426, 0580, 0687, 0691, and 0692 (identical haplotype collected from the same grower over a 4-year period). Alternatively, novel haplotypes (0310, 0317, 04016, 0582, 0690, 0785, 08223, 13084, and 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 to utilize 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.

In addition to 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

Fig. 5. Maximum-likelihood phylogenetic tree for the concatenated genes—*ligA*, *kdpA*, *sdhA*, and *tomA*—for reference *Clavibacter michiganensis* subsp. *sepedonicus* strain NCPPB2137 and *C. michiganensis* subsp. *michiganensis* isolates from New York, the United Kingdom, and Serbia ($n = 24$). Isolates from Serbia are designated with black dots (28), 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 (42).

enabled such a comparison due to the overlap in primer sets (28). 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 that 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, Milišašević-Marčić et al. (28) noted that bacterial canker outbreaks have gone unreported for approximately 50 years in Serbia, until large greenhouse outbreaks occurred around 2006 to 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 demonstrate that the New York population of *C. michiganensis* subsp. *michiganensis* is highly diverse, with 21 haplotypes observed over an 11-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, comprising five housekeeping genes and three putative pathogenicity 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 (14). 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-century of collections (14). It was interesting to observe a similar amount of diversity when just New York *C. michiganensis* subsp. *michiganensis* isolates from an 11-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-century resulted in a discriminatory power of just 0.800 (48).

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.

ACKNOWLEDGMENTS

This research was supported by grant number 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 number DGE-1144153. We thank K. Bekoscke and members of the Smart lab for assisting with various experiments.

LITERATURE CITED

- Balaji, V., Mayrose, M., Sherf, O., Jacob-Hirsch, J., Eichenlaub, R., Iraki, N., Manulis-Sasson, S., Rechavi, G., Barash, I., and Sessa, G. 2008. Tomato transcriptional changes in response to *Clavibacter michiganensis* subsp. *michiganensis* reveal a role for ethylene in disease development. *Plant Physiol.* 146:1797-1809.
- Balaji, V., and Smart, C. D. 2012. Over-expression of snakin-2 and extensin-like protein genes restricts pathogen invasiveness and enhances tolerance to *Clavibacter michiganensis* subsp. *michiganensis* in transgenic tomato (*Solanum lycopersicum*). *Transgenic Res.* 21:23-37.
- Bateman, A., Holden, M. T. G., and Yeats, C. 2005. The G5 domain: A potential N-acetylglucosamine recognition domain involved in biofilm formation. *Bioinformatics* 21:1301-1303.
- Bentley, S. D., Corton, C., Brown, S. E., Barron, A., Clark, L., Doggett, J., Harris, B., Ormond, D., Quail, M. A., May, G., Francis, D., Knudson, D., Parkhill, J., and Ishimaru, C. A. 2008. Genome of the actinomycete plant pathogen *Clavibacter michiganensis* subsp. *sepedonicus* suggests recent niche adaptation. *J. Bacteriol.* 190:2150-2160.
- Bryan, M. K. 1930. Studies on bacterial canker of tomato. *J. Agric. Res.* 41:825-851.
- Cannon, R., Niimi, K., Jenkinson, H., and Shepherd, M. 1994. Molecular cloning and expression of the *Candida albicans* beta-N-acetylglucosaminidase (HEX1) gene. *J. Bacteriol.* 176:2640-2647.
- Carlton, W. M., Braun, E. J., and Gleason, M. L. 1998. Ingress of *Clavibacter michiganensis* subsp. *michiganensis* into tomato leaves through hydathodes. *Phytopathology* 88:525-529.
- Chalupowicz, L., Cohen-Kandli, M., Dror, O., Eichenlaub, R., Gartemann, K.-H., Sessa, G., Barash, I., and Manulis-Sasson, S. 2010. Sequential expression of bacterial virulence and plant defense genes during infection of tomato with *Clavibacter michiganensis* subsp. *michiganensis*. *Phytopathology* 100:252-261.
- Chalupowicz, L., Zeller, M., Fluegel, M., Dror, O., Eichenlaub, R., Gartemann, K.-H., Savidor, A., Sessa, G., Iraki, N., Barash, I., and Manulis-Sasson, S. 2012. Colonization and movement of GFP-labeled *Clavibacter michiganensis* subsp. *michiganensis* during tomato infection. *Phytopathology* 102:23-31.
- Chen, Y., Zhang, W., and Knabel, S. J. 2007. Multi-virulence-locus sequence typing identifies single nucleotide polymorphisms which differentiate epidemic clones and outbreak strains of *Listeria monocytogenes*. *J. Clin. Microbiol.* 45:835-846.
- Chun, W. 1982. Identification and detection of *Corynebacterium michiganense* in tomato seed using the indirect enzyme-linked immunosorbent assay. M.Sc. thesis, University of Hawaii.
- Eichenlaub, R., and Gartemann, K.-H. 2011. The *Clavibacter michiganensis* subspecies: Molecular investigation of gram-positive bacterial plant pathogens. *Annu. Rev. Phytopathol.* 49:445-464.
- Gartemann, K.-H., Abt, B., Bekel, T., Burger, A., Engemann, J., Flügel, M., Gaigalat, L., Goesmann, A., Gräfen, I., Kalinowski, J., Kaup, O., Kirchner, O., Krause, L., Linke, B., McHardy, A., Meyer, F., Pohle, S., Rückert, C., Schneiker, S., Zeller, M., Pühler, A., Eichenlaub, R., Kaiser, O., and Bartels, D. 2008. The genome sequence of the tomato-pathogenic actinomycete *Clavibacter michiganensis* subsp. *michiganensis* NCPPB382 reveals a large island involved in pathogenicity. *J. Bacteriol.* 190:2138-2149.
- Jacques, M.-A., Durand, K., Orgeur, G., Balidas, S., Fricot, C., Bonneau, S., Quillévéry, A., Audusseau, C., Olivier, V., Grimault, V., and Mathis, R. 2012. Phylogenetic analysis and polyphasic characterization of *Clavibacter michiganensis* strains isolated from tomato seeds reveal that nonpathogenic strains are distinct from *C. michiganensis* subsp. *michiganensis*. *Appl. Environ. Microbiol.* 78:8388-8402.
- Jermyn, W. S., and Boyd, E. F. 2002. Characterization of a novel *Vibrio* pathogenicity island (VPI-2) encoding neuraminidase (nanH) among toxigenic *Vibrio cholerae* isolates. *Microbiology* 148:3681-3693.
- Jones, L. A. 2014. Prevalence and longitudinal study of human and plant pathogens in New York surface irrigation water, UV surface water treatment, and genome-assisted diagnostic protocol development for virulent field strains of *Pseudomonas syringae* pv. *tomato* 1-114. Ph.D. thesis, Cornell University, Geneva, NY.
- Kawaguchi, A., Tanina, K., and Inoue, K. 2010. Molecular typing and spread of *Clavibacter michiganensis* subsp. *michiganensis* in greenhouses in Japan. *Plant Pathol.* 59:76-83.
- Kleitman, F., Barash, I., Burger, A., Iraki, N., Falah, Y., Sessa, G., Weinthal, D., Chalupowicz, L., Gartemann, K.-H., Eichenlaub, R., and Manulis-Sasson, S. 2008. Characterization of a *Clavibacter michiganensis* subsp. *michiganensis* population in Israel. *Eur. J. Plant Pathol.* 121:463-475.
- Koressaar, T., and Remm, M. 2007. Enhancements and modifications of primer design program Primer3. *Bioinformatics* 23:1289-1291.
- Köser, C. U., Ellington, M. J., Cartwright, E. J. P., Gillespie, S. H., Brown, N. M., Farrington, M., Holden, M. T. G., Dougan, G., Bentley, S. D., Parkhill, J., and Peacock, S. J. 2012. Routine use of microbial whole genome sequencing in diagnostic and public health microbiology. *PLoS Pathog.* 8:e1002824.
- Kumar, M. J., Jamaluddin, M. S., Natarajan, K., Kaur, D., and Datta, A. 2000. The inducible N-acetylglucosamine catabolic pathway gene cluster in *Candida albicans*: Discrete N-acetylglucosamine-inducible factors interact at the promoter of NAG1. *Proc. Natl. Acad. Sci. USA* 97:14218-14223.
- De León, L., Rodríguez, A., Llop, P., López, M. M., and Siverio, F. 2009. Comparative study of genetic diversity of *Clavibacter michiganensis* subsp. *michiganensis* isolates from the Canary Islands by RAPD-PCR, BOX-PCR and AFLP. *Plant Pathol.* 58:862-871.
- De León, L., Siverio, F., López, M. M., and Rodríguez, A. 2011.

- Clavibacter michiganensis* subsp. *michiganensis*, a seedborne tomato pathogen: Healthy seeds are still the goal. *Plant Dis.* 95:1328-1338.
24. Louws, F. J., Bell, J., Medina-Mora, C. M., Smart, C. D., Opgenorth, D., Ishimaru, C. A., Hausbeck, M. K., de Bruijn, F. J., and Fulbright, D. W. 1998. rep-PCR-mediated genomic fingerprinting: A rapid and effective method to identify *Clavibacter michiganensis*. *Phytopathology* 88:862-868.
 25. Louws, F. J., Fulbright, D., Stephens, C., and de Bruijn, F. J. 1994. Specific genomic fingerprints of phytopathogenic *Xanthomonas* and *Pseudomonas* pathogens and strains generated with repetitive sequences and PCR. *Appl. Environ. Microbiol.* 60:2286-2295.
 26. Meletzus, D., Bermphol, A., Dreier, J., and Eichenlaub, R. 1993. Evidence for plasmid-encoded virulence factors in the phytopathogenic bacterium *Clavibacter michiganensis* subsp. *michiganensis* NCPPB382. *J. Bacteriol.* 175:2131-2136.
 27. Meletzus, D., and Eichenlaub, R. 1991. Transformation of the phytopathogenic bacterium *Clavibacter michiganense* subsp. *michiganense* by electroporation and development of a cloning vector. *J. Bacteriol.* 173:184-190.
 28. Milijašević-Marčić, S., Gartemann, K., Frohwitter, J., Eichenlaub, R., Todorovic, B., Rekanovic, E., and Potocnik, I. 2012. Characterization of *Clavibacter michiganensis* subsp. *michiganensis* strains from recent outbreaks of bacterial wilt and canker in Serbia. *Eur. J. Plant Pathol.* 134:697-711.
 29. Nazari, F., Niknam, G. R., Ghasemi, A., Taghavi, S. M., Momeni, H., and Torabi, S. 2007. An investigation on strains of *Clavibacter michiganensis* subsp. *michiganensis* in north and north west of Iran. *J. Phytopathol.* 155:563-569.
 30. Peakall, R., and Smouse, P. E. 2006. GENALEX 6: Genetic analysis in Excel. Population genetic software for teaching and research. *Mol. Ecol. Notes* 6:288-295.
 31. Peakall, R., and Smouse, P. E. 2012. GenA1Ex 6.5: Genetic analysis in Excel. Population genetic software for teaching and research-an update. *Bioinformatics* 28:2537-2539.
 32. Qian, W., Jia, Y., Ren, S.-X., He, Y.-Q., Feng, J.-X., Lu, L.-F., Sun, Q., Ying, G., Tang, D.-J., Tang, H., Wu, W., Hao, P., Wang, L., Jiang, B.-L., Zeng, S., Gu, W.-Y., Lu, G., Rong, L., Tian, Y., Yao, Z., Fu, G., Chen, B., Fang, R., Qiang, B., Chen, Z., Zhao, G.-P., Tang, J. L., and He, C. 2005. Comparative and functional genomic analyses of the pathogenicity of phytopathogen *Xanthomonas campestris* pv. *campestris*. *Genome Res.* 15:757-767.
 33. Quesada-Ocampo, L., Landers, N., Lebeis, A., Fulbright, D., and Hausbeck, M. 2012. Genetic structure of *Clavibacter michiganensis* subsp. *michiganensis* populations in Michigan commercial tomato fields. *Plant Dis.* 96:788-796.
 34. Reid, S. D., Green, N. M., Buss, J. K., Lei, B., and Musser, J. M. 2001. Multilocus analysis of extracellular putative virulence proteins made by group A *Streptococcus*: Population genetics, human serologic response, and gene transcription. *Proc. Natl. Acad. Sci. USA* 98:7552-7557.
 35. Richert, K., Brambilla, E., and Stackebrandt, E. 2005. Development of PCR primers specific for the amplification and direct sequencing of *gyrB* genes from microbacteria, order *Actinomycetales*. *J. Microbiol. Methods* 6:115-123.
 36. Savidor, A., Teper, D., Gartemann, K.-H., Eichenlaub, R., Chalupowicz, L., Manulis-Sasson, S., Barash, I., Tews, H., Mayer, K., Giannone, R. J., Hettich, R. L., and Sessa, G. 2012. The *Clavibacter michiganensis* subsp. *michiganensis*-tomato interactome reveals the perception of pathogen by the host and suggests mechanisms of infection. *J. Proteome Res.* 11:736-750.
 37. Schneider, K. L., Marrero, G., Alvarez, A. M., and Presting, G. G. 2011. Classification of plant associated bacteria using RIF, a computationally derived DNA marker. *PLoS One* 6:e18496.
 38. Sen, Y., Feng, Z., Vandenbroucke, H., van der Wolf, J., Visser, R. G. F., and van Heusden, A. W. 2013. Screening for new sources of resistance to *Clavibacter michiganensis* subsp. *michiganensis* (Cmm) in tomato. *Euphytica* 190:309-317.
 39. Shariat, N., Sandt, C. H., DiMarzio, M. J., Barrangou, R., and Dudley, E. G. 2013. CRISPR-MVLST subtyping of *Salmonella enterica* subsp. *enterica* serovars Typhimurium and Heidelberg and application in identifying outbreak isolates. *BMC Microbiol.* 13:254-271.
 40. Stork, I., Gartemann, K., Burger, A., and Eichenlaub, R. 2008. A family of serine proteases of *Clavibacter michiganensis* subsp. *michiganensis*: chpC plays a role in colonization of the host plant tomato. *Mol. Plant Pathol.* 9:599-608.
 41. Tamura, K., and Nei, M. 1993. Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. *Mol. Biol. Evol.* 10:512-526.
 42. Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., and Kumar, S. 2011. MEGA 5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol. Biol. Evol.* 28:2731-2739.
 43. 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. *Appl. Environ. Microbiol.* 79:6948-6957.
 44. Untergrasser, A., Cutcutache, I., Koressaar, T., Ye, J., Faircloth, B., Remm, M., and Rozen, S. 2012. Primer3—New capabilities and interfaces. *Nucleic Acids Res.* 40:e115.
 45. Werner, N., Fulbright, D., Podolsky, R., Bell, J., and Hausbeck, M. K. 2002. Limiting populations and spread of *Clavibacter michiganensis* subsp. *michiganensis* on seedling tomatoes in the greenhouse. *Plant Dis.* 86:535-542.
 46. Wicker, E., Lefeuvre, P., de Cambiaire, J.-C., Lemaire, C., Poussier, S., and Prior, P. 2012. Contrasting recombination patterns and demographic histories of the plant pathogen *Ralstonia solanacearum* inferred from MLSA. *ISME J.* 6:961-974.
 47. Yasuhara-Bell, J., Kubota, R., Jenkins, D. M., and Alvarez, A. M. 2013. Loop-mediated amplification of the *Clavibacter michiganensis* subsp. *michiganensis* *micA* gene is highly specific. *Phytopathology* 103:1220-1226.
 48. Zaluga, J., Stragier, P., Van Vaerenbergh, J., Maes, M., De Vos, P., Vaerenbergh, J. Van, and Vos, P. De. 2013. Multilocus variable-number-tandem-repeats analysis (MLVA) distinguishes a clonal complex of *Clavibacter michiganensis* subsp. *michiganensis* strains isolated from recent outbreaks of bacterial wilt and canker in Belgium. *BMC Microbiol.* 13:126-141.
 49. Zhang, W., Jayarao, B., and Knabel, S. 2004. Multi-virulence-locus sequence typing of *Listeria monocytogenes*. *Appl. Environ. Microbiol.* 70:913-920.