Comparative Genomics to Develop a Specific Multiplex PCR Assay for Detection of *Clavibacter michiganensis*

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ABSTRACT

Clavibacter michiganensis is a Gram-positive bacterial pathogen that proliferates in the xylem vessels of tomato, causing bacterial wilt and canker symptoms. Accurate detection is a crucial step in confirming outbreaks of bacterial canker and developing management strategies. A major problem with existing detection methods are false-positive and -negative results. Here, we report the use of comparative genomics of 37 diverse *Clavibacter* strains, including 21 strains sequenced in this study, to identify specific sequences that are *C. michiganensis* detection targets. Genome-wide phylogenetic analyses revealed additional diversity within the genus *Clavibacter*. Pathogenic *C. michiganensis* strains varied in plasmid composition, highlighting the need for detection methods based on chromosomal targets. We utilized sequences of *C. michiganensis*-specific loci to develop a multiplex PCR-based diagnostic platform using

two *C. michiganensis* chromosomal genes (*rhuM* and *tomA*) and an internal control amplifying both bacterial and plant DNA (16S ribosomal RNA). The multiplex PCR assay specifically detected *C. michiganensis* strains from a panel of 110 additional bacteria, including other *Clavibacter* spp. and bacterial pathogens of tomato. The assay was adapted to detect the presence of *C. michiganensis* in seed and tomato plant materials with high sensitivity and specificity. In conclusion, the described method represents a robust, specific tool for detection of *C. michiganensis* in tomato seed and infected plants.

Keywords: bacteriology, *Clavibacter michiganensis*, comparative genomics, diagnostic, disease control and pest management, multiplex PCR, techniques, tomato

Clavibacter spp. are xylem colonizing Gram-positive bacteria that can infect both monocots and dicots. Multiple *Clavibacter* subspecies have been recently elevated to the species level based on average nucleotide identities derived from whole-genome analyses and digital DNA-DNA hybridization (Li et al. 2018). *Clavibacter michiganensis* causes bacterial canker and is one of the most important bacterial diseases of tomato (de León et al. 2011; Mansfield et al. 2012). *C. michiganensis* gains entry into the plant through contaminated seed, wounds, broken trichomes, or hydathodes, and then proliferates in xylem vessels (Eichenlaub et al. 2006; Sen et al. 2015). Systemic infection results in unilateral leaf wilting, necrosis of the entire plant, necrosis of stems and petioles, as well as formation of cankers (de León et al. 2011). *C. michiganensis* can also form localized infections, resulting in fruit-spotting “bird’s eye” and blister-like spots on vegetative tissues (de León et al. 2011).

Bacterial canker can cause substantial losses in greenhouse and field production systems. Since the first report of the disease in Michigan in 1910, it has spread throughout the world (Smith 1910). The economic threat and the challenges to manage this disease have led to the inclusion of this pathogen as a quarantine agent in Europe, Asia, Africa, and the Caribbean (Anonymous 2000; Eichenlaub and Gartemann 2011). Despite the importance of this disease, there

currently are no resistant commercial cultivars or effective chemical control strategies. At present, control and management of this disease rely primarily on the use of clean seed, healthy transplant practices, sanitation, and crop rotation.

Effective disease management also requires precise, reliable, and sensitive detection methods. Historically, methods are based on semiselective media followed by pathogenicity tests (Fatmi and Schaad 2002; Fatmi et al. 2017). These diagnostic assays are expensive, require laboratory and greenhouse facilities, and are unsuitable for large-scale screening. Current *C. michiganensis* detection strategies mainly utilize serological (Franken et al. 1993) or PCR-based methods, with primers targeting different loci, often followed by bacterial isolation on semiselective media (Dreier et al. 1995; Kaneshiro et al. 2006; Louws et al. 1998; Milišašević-Marčić et al. 2012). However, the current serological techniques such as enzyme-linked immunosorbent assay and immunostrips are not reliable due to their cross reaction with other *Clavibacter* spp. (Dreier et al. 1995; Franken et al. 1993; Jacques et al. 2012).

The presence of closely related *Clavibacter* endophytes in tomato complicates precise detection of *C. michiganensis*, making it difficult to differentiate pathogenic *C. michiganensis* from other *Clavibacter* spp. Nonpathogenic *Clavibacter* spp. have been frequently found associated with tomato plants and seed (Jacques et al. 2012; Thapa et al. 2017; Yasuhara-Bell and Alvarez 2015; Zaluga et al. 2013). Furthermore, analyses of *C. michiganensis* strains collected from diverse geographical origins have revealed considerable genetic diversity, particularly with respect to plasmid composition, plasmid number, and the presence or absence of putative chromosomally encoded pathogenicity genes (Jacques et al. 2012; Milišašević-Marčić et al. 2012; Tancos et al. 2015; Thapa et al. 2017). The presence of endophytic *Clavibacter* spp. in tomato and the diversity in *C. michiganensis* highlights the need for a rapid, specific, and accurate method for detecting *C. michiganensis* from seed, plants, and other substrates.

PCR is rapid and highly sensitive and can be specific for identification of bacterial pathogens (McNally et al. 2016; Schaad

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and Frederick 2002; Vincelli and Tisserat 2008). Several pairs of primers have been designed for detecting *C. michiganensis* (Alvarez et al. 2005; Kaneshiro et al. 2006; Kleitman et al. 2008; Santos et al. 1997). However, most of these primers target genes present in plasmids (pCM1 and pCM2) and the *chp/tomA* pathogenicity island based on the reference strain NCPPB382 (Alvarez et al. 2005; Kaneshiro et al. 2006; Kleitman et al. 2008). False-positive and -negative results have been reported with most primers, and different *C. michiganensis* strains can possess variable plasmid components yet retain similar virulence (Alvarez et al. 2005; Kleitman et al. 2008; Louws et al. 1998; Tancos et al. 2015; Thapa et al. 2017). Therefore, there is a need for a robust diagnostic assay for *C. michiganensis* capable of targeting multiple genes that can differentiate *C. michiganensis* from other *Clavibacter* spp. and bacterial pathogens associated with tomato.

Multiplex PCR (mPCR) can be used to amplify multiple genetic loci, facilitating detection of several targets in a single pathogen (Bertolini et al. 2003; Ozdemir 2009; Thapa et al. 2012, 2013). Here, we used comparative genomics to analyze a wide range of strains representing the genus *Clavibacter*. This analysis revealed diversity among *C. michiganensis* strains, including multiple subgroups and variable plasmid components. Genome analyses were used to identify highly conserved mPCR targets within *C. michiganensis* strains. We have also validated the specificity and sensitivity of this assay on vegetative and seed samples using diverse *C. michiganensis* strains, including those collected from a recent outbreak in California.

MATERIALS AND METHODS

Bacterial strains and DNA extraction. The bacterial strains used in this study are listed in Supplementary Table S1. *C. michiganensis* strains were grown in tryptone broth with yeast (TBY) and on D2 semiselective media at 28°C (Kirchner et al. 2001). For DNA extraction, overnight cultures were grown in TBY broth at 28°C on a rotary shaker at 200 rpm. DNA was extracted from infected plant materials or bacterial cultures for PCR using either a crude lysis method or cetyltrimethylammonium bromide (CTAB) purification (Murray and Thompson 1980). For the lysis method, 100 mg of plant material or bacterial cells was resuspended in extraction buffer (Na₂HPO₄ at 7.75 g/liter, KH₂PO₄ at 1.65 g/liter, and Tween 20 at 0.2 ml/liter). The supernatant was then added to cell lysis buffer (3% sodium dodecyl sulfate, 50 µM Tris, and 10% sucrose) and incubated at 100°C in heat block for 15 min. The samples were centrifuged at 11,000 × g for 5 min. The supernatant was transferred into new Eppendorf tubes. DNA was precipitated with 2× volume of 100% ethanol and centrifuged at 11,000 × g for 5 min, and the supernatant was discarded. The pellet was then washed with 70% ethanol, air dried, mixed in distilled H₂O (dH₂O), and used for mPCR analyses. CTAB DNA extraction was performed as previously described (Murray and Thompson 1980).

Genome sequencing, assembly, and annotation. *C. michiganensis* strains sequenced in the present study are shown in Table 1. Total genomic DNA was isolated with the Promega Wizard Genomic DNA Purification Kit. For library construction for the Illumina platform, genomic DNA was sheared with a Covaris E220 sonicator, followed by end repair and phosphorylation. Fragmented DNA was used to construct paired-end libraries. Libraries were checked for size with a High Sensitivity DNA Kit (Agilent) in the Bioanalyzer 2100, with an expected average size of 250 bp. Libraries were pooled and sequenced with paired-end reads on the HiSeq 2500 system (Illumina). Genome sequencing was performed at the University of California–Davis DNA Technologies Core Facility. Genomes sequenced on Illumina were de novo assembled with the SPAdes (Bankevich et al. 2012). The draft genomes were annotated with Prokka and the NCBI Prokaryotic Genome Annotation Pipeline (Seemann 2014; Tatusova et al. 2016).

Phylogenetic analyses. Orthologous genes of *Clavibacter* strains were predicted with the OrthoMCL pipeline (Li et al. 2003). All-versus-all BLASTN (*E* value < 1 × 10⁻⁵, alignment coverage > 50%) comparison of all gene sequences for each species was performed, and orthologous genes were clustered with OrthoMCL (Li et al. 2003). The normalized scores were fed into the MCL algorithm to classify the genes into predicted orthologous gene families with a default inflation parameter of 1.5. We performed multiple alignments of gene sequences with Prank (Loytynoja and Goldman 2005). Alignments were concatenated by FASconCAT, yielding a gene supermatrix (Kück and Meusemann 2010). A maximum-likelihood approach was used to reconstruct the phylogenetic tree using RAXML software (Stamatakis 2006). Bootstrapping was performed with 1,000 replicates. The resulting phylogeny was visualized with FigTree (Rambaut 2012).

Comparative genomics and primer design. Comparative analysis was performed using 145,929 genes from 21 sequenced genomes (the present study) and 24 published *Clavibacter* genomes. In order to identify gene conservation across *C. michiganensis*, we performed whole-genome clustering analyses based on sequence identity using CD-HIT software with an identity threshold set to 95% (Li and Godzik 2006). Primers were designed based upon conserved sequences that were present only in *C. michiganensis* and absent in other *Clavibacter* or bacterial strains. Primers were designed based on specificity, amplicon size, and compatibility in mPCR using Primer3 (<http://bioinfo.ut.ee/primer3-0.4.0/>). Primer design was optimized to minimize secondary structure, and to have compatible annealing temperature and minimal cross dimerization. Primer specificity was tested in silico by a similarity search against the NCBI sequence database (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>). Three primer sets, two *C. michiganensis* specific and one internal control, were designed to amplify RhuM (CMM_2694), TomA (CMM_0090), and 16S (CMM_RNA_0001) (Supplementary Table S2).

mPCR assay and limit of detection. mPCR was performed with RhuM-F/R, TomA-F1/R1, and 16sR-F/R primer pairs. The specificity of the mPCR assay was determined by using 75 *C. michiganensis*, 11 *C. sepedonicus*, 1 *C. insidiosus*, 2 *C. tessellarius*, 6 endophytic *Clavibacter* strains, and 15 other bacterial pathogens of tomato (Supplementary Table S1). Total genomic DNA was isolated with the Promega Wizard Genomic DNA Purification Kit. Each primer combination was tested using the C1000 Touch Thermal Cycler (Bio-Rad, Hercules, CA, U.S.A.). mPCR was performed in 25 µl of final volume with 1 to 4 µl of DNA; 0.7 µM (RhuM-F/R), 0.5 µM (TomA-F1/R1), and 0.3 µM (16sR-F/R) primers; 4 µl of GC buffer, and iProof polymerase (Bio-Rad) at 0.02 U/µl. A temperature gradient of 58 to 65°C was used to determine the optimum annealing temperature. All PCR amplifications were carried out in a C1000 Touch Thermal Cycler (Bio-Rad). Final PCR conditions were as follows: initial denaturation of 98°C for 2 min; followed by 30 cycles at 98°C for 30 s, 60°C for 30 s, and 68°C for 1 min; and a final extension step of 68°C for 7 min. mPCR products were electrophoresed for 30 min at 130 V on a 1% agarose gels.

To evaluate the sensitivity of the mPCR assay, bacterial cultures and genomic DNA of *C. michiganensis* CASJ002 were serially diluted and used as a template. For DNA, 10 ng of total *C. michiganensis* genomic DNA was diluted and used for mPCR assay in a C1000 Touch Thermal Cycler (Bio-Rad) following conditions as described above. For bacterial cultures, *C. michiganensis* (CASJ002) was grown on TBY broth and cells were serially diluted followed by DNA extraction using the lysis method. Each of the dilutions (100 µl) was spread on TBY and incubated for 48 h at 28°C to determine the concentration of cells in the suspension.

Plant growth and pathogenicity assays. Tomato cultivar Early Pak 7 (EP-7) was grown in the greenhouse at 23°C and 50% relative humidity with a 14-h photoperiod and supplemental lighting as necessary (Thapa et al. 2015). Tomato plants (3 to 4 weeks old) were used for inoculation assays. Tomato plants were inoculated

with *C. michiganensis* strain CASJ002 as previously described (Thapa et al. 2017). To obtain DNA from inoculated plants, samples were collected 7 and 14 days postinoculation. Stem segments (1 cm each) were cut at 1, 3, 6, 9, and 15 cm above the inoculation site and from apical regions. Stem segments were surface disinfected in 75% ethanol, the outer epidermis was removed, and the remaining segment was ground in 500 µl of dH₂O. Then, 100 µl of the resulting supernatant was used for DNA extraction using CTAB, and 100 µl was used for determination of bacterial titers.

Seed infestation, sampling, and DNA extraction. For seed contamination, CASJ002 inoculum was prepared as described above. Inoculum was prepared by diluting cells in 10 mM MgCl₂ to a concentration of approximately 10⁷ CFU/ml. EP-7 seed lots (10 g) were then incubated with 40 ml of *C. michiganensis* CASJ002 in H₂O for 24 h at 37°C. After 24 h, the liquid was removed and seed were dried at 24°C on filter paper for 2 days and kept for 2 to 5 weeks at room temperature. The detection threshold was evaluated by adding different numbers of contaminated seed to clean seed samples of 10,000 seeds. Samples of 10,000 seeds (approximately 30 g) were suspended in 120 ml of sterile phosphate-buffered saline buffer. The samples were incubated at 4°C for at least 12 h, shaken for 2 to 3 h at room temperature, then transferred to whirl pack bags (Whirl-pak Nasco, Modesto, CA, U.S.A.) and processed in a

stomacher (Stomacher 400, Seward, England) for 20 min. Both the lysis and CTAB procedure were used for the isolation of DNA to be used for mPCR. The PCR conditions used were as described above.

Collection of field samples. During 2016, 21 samples of field-grown tomato plants exhibiting bacterial canker-like symptoms were collected from the California Central Valley (Supplementary Table S3). Stem and leaf samples of plants exhibiting disease symptoms were used for DNA extraction and bacterial isolation. DNA was extracted from 1 mg of plant materials using both lysis and CTAB procedures and used in mPCR tests. *C. michiganensis* isolation was performed using the semiselective D2 media (Kado and Heskett 1970).

Accession numbers. Whole-genome sequences of strains sequenced in this study were submitted to NCBI under the accession numbers listed in Table 1.

RESULTS

***C. michiganensis* genome sequencing and phylogenomics.** We sequenced 21 *C. michiganensis* genomes from strains isolated from different locations worldwide using the Illumina platform (Supplementary Fig. S1A). These 21 *C. michiganensis* strains were

TABLE 1. Genome statistics and GenBank accession numbers of *Clavibacter* genomes analyzed in this study

Species	Strains ^a	Source	Contigs (n)	Note	Genome size (bp)	Accession number
<i>Clavibacter michiganensis</i>	ARZ28	United States	434	This study	3,281,597	QLNE000000000
<i>C. michiganensis</i>	ATCC10202	...	334	This study	3,275,372	QLMX000000000
<i>C. michiganensis</i>	ATCC14456	Italy	41	This study	3,296,963	QLMU000000000
<i>C. michiganensis</i>	CFBP1465	France	66	This study	3,328,034	QLMV000000000
<i>C. michiganensis</i>	CFBP1940	Spain	35	This study	3,296,592	QLMW000000000
<i>C. michiganensis</i>	CFBP2494	Algeria	39	This study	3,316,980	QLMY000000000
<i>C. michiganensis</i>	CFBP2500	Algeria	332	This study	3,305,560	QLMZ000000000
<i>C. michiganensis</i>	CFBP5842	Brazil	127	This study	3,343,022	QLNA000000000
<i>C. michiganensis</i>	CFBP6885	France	25	This study	3,327,889	QLML000000000
<i>C. michiganensis</i>	CFBP7158	New Zealand	23	This study	3,285,302	QLNB000000000
<i>C. michiganensis</i>	CFBP7311	Morocco	46	This study	3,301,380	QLNC000000000
<i>C. michiganensis</i>	CFBP7312	China	43	This study	3,319,896	QLND000000000
<i>C. michiganensis</i>	CFBP7314	United States	30	This study	3,268,866	QLMM000000000
<i>C. michiganensis</i>	CFBP7315	United States	31	This study	3,311,336	QLMN000000000
<i>C. michiganensis</i>	CFBP7316	United States	29	This study	3,258,979	QLMO000000000
<i>C. michiganensis</i>	CFBP7488	France	55	This study	3,299,275	QLMP000000000
<i>C. michiganensis</i>	CFBP7568	United States	61	This study	3,347,875	QLMQ000000000
<i>C. michiganensis</i>	CFBP7589	Belgium	35	This study	3,316,741	QLMR000000000
<i>C. michiganensis</i>	NZ1811	New Zealand	333	This study	3,273,907	QLMS000000000
<i>C. michiganensis</i>	NZ5026	United States	263	This study	3,283,188	QLMT000000000
<i>C. michiganensis</i>	NZ2541	United Kingdom	292	This study	3,321,838	QODA000000000
<i>C. michiganensis</i>	CASJ001	United States	4	GenBank	3,423,339	MDHB000000000
<i>C. michiganensis</i>	CASJ002	United States	3	GenBank	3,423,641	MDHC000000000
<i>C. michiganensis</i>	CASJ003	United States	315	GenBank	3,294,503	MDHD000000000
<i>C. michiganensis</i>	CASJ004	United States	477	GenBank	3,351,833	MDHE000000000
<i>C. michiganensis</i>	CASJ005	United States	321	GenBank	3,284,480	MDHF000000000
<i>C. michiganensis</i>	CASJ006	United States	4	GenBank	3,499,162	MDHG000000000
<i>C. michiganensis</i>	CASJ007	United States	11	GenBank	3,400,049	MDHH000000000
<i>C. michiganensis</i>	CASJ008	United States	39	GenBank	3,392,817	MDHI000000000
<i>C. michiganensis</i>	LMG7333 ^T	Hungary	5	GenBank	3,391,512	NZ_MZMP000000000.1
<i>C. michiganensis</i>	VKMAc	Hungary	13	GenBank	3,295,160	NZ_FVZG000000000.1
<i>C. michiganensis</i>	CAYO001	United States	3	GenBank	3,481,307	MDHL000000000
<i>C. michiganensis</i>	CA00001	United States	120	GenBank	3,625,805	MDHK000000000
<i>C. michiganensis</i>	CA00002	United States	4	GenBank	3,371,744	MDHM000000000
<i>C. michiganensis</i>	NCPPB382	United Kingdom	...	GenBank	3,395,240	AM711867.1
<i>C. sepedonicus</i>	ATCC33113	...	1	GenBank	3,258,645	NC_010407.1
<i>C. insidiosus</i>	LMG3663	United States	3	GenBank	3,387,165	NZ_MZMO000000000.1
<i>C. capcisi</i>	PF008	South Korea	3	GenBank	3,056,296	NZ_CP012573
<i>C. tessellarius</i>	ATCC33566	United States	2	GenBank	3,318,535	MZMQ000000000.1
<i>C. nebraskensis</i>	NCPPB2581	United States	1	GenBank	3,063,596	NC_020891.1
<i>Clavibacter</i> spp.	CFBP7494	Chile	15	GenBank	3,425,817	MDJW000000000
<i>Clavibacter</i> spp.	CFBP7576	...	372	GenBank	3,399,000	MDJX000000000
<i>Clavibacter</i> spp.	CFBP8017	The Netherlands	65	GenBank	3,284,576	MDJY000000000
<i>Clavibacter</i> spp.	CFBP8019	The Netherlands	18	GenBank	3,076,288	MDJZ000000000
<i>Clavibacter</i> spp.	LMG26808	The Netherlands	70	GenBank	3,420,753	NZ_AZQZ01000000

^a ATCC = American Type Culture Collection, CFBP = French Collection of Plant-associated Bacteria, LMG = Laboratorium voor Microbiologie, and superscript T = type strain.

verified to be pathogenic on tomato after inoculation of the cultivar EP-7 and were able to induce leaflet necrosis, wilting, and stem canker symptoms (Fig. 1). Genomes were sequenced on the Illumina platform, de novo assembled with the SPAdes, and annotated with Prokka and the NCBI Prokaryotic Genome Annotation Pipeline (Table 1). To facilitate a high-resolution comparison of *Clavibacter* spp., the core genome of all 21 strains sequenced in the present study and 16 other published genomes, including five *Clavibacter* spp. pathogenic on other crops and five tomato endophytes, were analyzed. Using the orthoMCL pipeline, we identified a total of 1,258 orthologous genes that are conserved between different *Clavibacter* spp. (Supplementary Fig. S2). The 1,258 orthologous genes were concatenated and a maximum-likelihood approach was employed to generate a *Clavibacter* phylogeny (Fig. 1). *C. michiganensis* strains formed a monophyletic clade strongly supported by a high bootstrap value (100%) and clearly separated from other *Clavibacter* spp. The phylogenetic analyses also refined the genetic relationship among the *C. michiganensis* strains (Fig. 1). The relatedness among *C. michiganensis* strains does not correlate with geographical location or year of isolation. This is likely due to the global production and distribution of tomato seed, making it extremely hard to trace geographic origins of strains or sources. Collectively, these data demonstrate that *C. michiganensis* strains are not clonal

and support the concept of multiple independent introductions, consistent with seedborne disease.

All *Clavibacter* genomes contained one circular chromosome approximately 3.2 Mb in size, encoding an average of approximately 3,200 protein-coding sequences per genome (Table 1; Supplementary Fig. S1B and C). Plasmids with similarity to pCM1 and pCM2, which are found in reference strain NCPPB382, were detected based on similarity in their plasmid backbone (replication or partitioning genes) and conjugal transfer genes. *C. michiganensis* strains possessed at least one virulence plasmid of the pCM1 or pCM2 type (Fig. 1) (Meletzus et al. 1993). Of the 34 *C. michiganensis* genomes analyzed, 33 possessed pCM1-like plasmids, while 29 possessed pCM2-like plasmids (Fig. 1). Both plasmids are required for virulence in NCPPB382 (Meletzus et al. 1993). However, we have identified multiple *C. michiganensis* strains with only pCM2 that retain pathogenicity (Thapa et al. 2017) (Fig. 1). In addition, the *C. michiganensis* strain NZ2541 lacks pCM1 but is still pathogenic on tomato (Figs. 1 and 2). We compared the genomes of reference *C. michiganensis* strain NCPPB382 with strains NZ2541, which lacks pCM1, and CASJ001, which lacks pCM2. Genome visualizations were performed using circos (Supplementary Fig. S1B and C) (Kryzysinski et al. 2009). Although the plasmid components of *C. michiganensis* strains NCPPB382, NZ2541, and CASJ001 differ, they display a high level of chromosomal sequence similarity

Fig. 1. Genome-assisted phylogeny of the genus *Clavibacter*. In total, 1,258 orthologous genes from each strain were concatenated. A maximum-likelihood approach was used to generate the phylogeny with 1,000 bootstrap replicates. Bootstrap values are indicated at each node. Columns indicate the presence of the pCM1 plasmid, the pCM2 plasmid, the *chp/tomA* genomic island, and *rhuM*. Phenotype = shading indicates pathogenicity on tomato, Host = plants bacteria were isolated from if known, and E = endophyte of tomato.

(Supplementary Fig. S1B and C). We also detected some instances of genome rearrangement between *C. michiganensis* strains, including CASJ001 and NZ2541 (Supplementary Fig. S1B and C).

Although there is significant variation within pCM1-like plasmids, the *celA* gene is conserved in the pCM1 plasmids of all *C. michiganensis* strains analyzed (Fig. 1). Previously, it was demonstrated that *celA* is required for the induction of bacterial wilt of tomato in NCPPB382 and CASJ002 (Jahr et al. 2000; Thapa et al. 2017). However, NZ2541 also causes canker symptoms on tomato and grows to levels similar to those of NCPPB382 and CASJ002, even though it lacks pCM1 and *celA* (Fig. 2). Strain NZ2541 contains pCM2 in addition to a novel plasmid that we have named pCM3 (Fig. 2A). These data highlight the genetic variation present in *C. michiganensis* plasmid types.

Comparative genomics and design of *C. michiganensis*-specific primers. Our genomic analyses of diverse *Clavibacter* strains indicated that several of the existing *C. michiganensis*-specific primers were likely nonspecific, considering the differences in plasmid content and composition (Supplementary Table S2). We analyzed eight primer pairs for specificity in detecting *C. michiganensis*: PFC3/5, P5/6, ppA, *tomA*, *celA*, *Cmm3/4*, *Cmm5/6*, and *PSA* (Dreier et al. 1995; Jahr et al. 2000; Kleitman et al. 2008; Pastrik and Rainey 1999; Santos et al. 1997). In order to determine the specificity of commonly used published primers, we tested them on a panel of 110 bacterial strains, including 75 *C. michiganensis* strains, 20 other *Clavibacter* strains, 6 *Pseudomonas* spp., and 9 *Xanthomonas* spp. (Supplementary Table

S1 and S2). Of the eight tested primer pairs, only the *tomA*-F/R pair, amplifying the *tomA* tomatinase on the *chp/tomA* genomic island, specifically detected all pathogenic *C. michiganensis* strains but not other *Clavibacter* spp. or subspecies (Supplementary Table S2).

Given the genetic diversity of *C. michiganensis* and variation in plasmid types, we sought to identify multiple genetic loci conserved among and specific to *C. michiganensis*. To identify *C. michiganensis*-specific loci, we performed whole-genome comparisons of 145,929 genes from 45 *Clavibacter* genomes. We identified *C. michiganensis*-specific loci and tested 10 primer pairs whose amplicons could be separated by size by gel electrophoreses after amplification in a single mPCR assay. From these primer pairs, two targets were chosen: *rhuM* (hypothetical protein) (CMM_2694) and *tomA* (tomatinase) (CMM_0090) due to their high amplification efficiency (Figs. 1 and 3A). The sequences of these genes were compared individually to the nr and the microbial genome databases of the NCBI BLAST web site to confirm their specificity. These results revealed that *tomA* and *rhuM* are highly conserved among *C. michiganensis* and similar nucleotide sequences are not found in other bacteria (Fig. 3). The amplification specificity of the *tomA* and *rhuM* primer pairs was confirmed in PCR tests with DNA of a bacterial panel by PCR (Supplementary Table S1).

The mPCR assay targeted three loci, two of which are *C. michiganensis* specific (*tomA* and *rhuM*) and a third amplifying a region of the 16S ribosomal RNA (rRNA) in bacteria and plant mitochondria that serves as an internal positive control. Primer sets were designed to possess similar melting temperatures (58 to

Fig. 2. *Clavibacter michiganensis* strain NZ2541 lacks the pCM1 plasmid and *celA* but is pathogenic on tomato. **A**, Comparison of NCPPB382's pCM1 plasmid with the new plasmid found in NZ2541, pCM3. **B**, Representative bacterial wilt and canker symptoms induced by *C. michiganensis* strain in tomato 7 days postinoculation (dpi). **C**, Bacterial titers in infected tomato stems 7 dpi. Three-week-old tomato plants (*Solanum lycopersicum* 'Early Pak 7') were inoculated by stabbing tomato stems with a needle and delivering 5 μ l of bacteria at 10^7 CFU/ml. Bacterial titers were determined by grinding sterilized stem segments 7 dpi and plating on D2 semiselective media. Statistical differences were detected by ANOVA followed by Tukey's HSD. Asterisks indicate $P = 0.001$.

63°C), direct the amplification of the conserved *C. michiganensis* sequences, and produce PCR product sizes so that the amplicons could be readily distinguished. The primer sets directed the amplification of a 1,000-bp *rhuM* fragment, a 630-bp *tomA* fragment, and a 415-bp 16S rRNA fragment (Fig. 3B).

Validation of the *C. michiganensis* mPCR assay. The specificity of the mPCR assay was determined by analyzing 75 *C. michiganensis* strains, 20 other *Clavibacter* strains, and 15 non-*Clavibacter* spp. (Supplementary Table S1). The RhuMF/R and TomA-F1/R1 primer sets directed the amplification of the target sequences from *C. michiganensis* strains but not from DNA of other *Clavibacter* spp. or other bacteria (Fig. 3B). The mPCR amplified the three expected size fragments of 1,000 bp (*rhuM*), 630 bp (*tomA*), and 415 bp (16S rRNA) when purified *C. michiganensis* DNA was used as a template (Fig. 3B). No false positives were detected with *Clavibacter* endophytes, *Clavibacter* pathogens on other crops, or other bacterial pathogens of tomato, demonstrating the specificity of the mPCR assay (Figs. 3B and 4B). In contrast, the

415-bp 16S rRNA fragment was amplified from tomato and all bacterial strains tested, validating the utility of the internal positive control. The identity of selected PCR products was confirmed by sequencing.

We next evaluated the limit of detection of the mPCR assay. The detection limit was 0.01 ng with CTAB-purified genomic DNA from *C. michiganensis* strain CASJ002 grown in pure culture (Supplementary Fig. S3A). We also analyzed the ability to detect *C. michiganensis* after serially diluting cultures of *C. michiganensis* strain CASJ002 followed by simple DNA extraction using the lysis method and were able to reliably detect 500 cells (Supplementary Fig. S3B). Similar detection levels were found for *C. michiganensis* strain NCPPB382 with both extraction methods (data not shown).

mPCR can detect *C. michiganensis* in contaminated plant and seed samples. To determine the capacity of the mPCR assay for detection of *C. michiganensis* in planta, we extracted DNA with the CTAB method from inoculated tomato plants (cultivar EP-7) under greenhouse conditions. Samples were collected at 7

Fig. 3. *Clavibacter michiganensis* primer design and multiplex PCR. **A**, Genetic architecture of flanking genes surrounding the *tomA* and *rhuM* loci. The *chp*/*tomA* cluster, including *tomA*, is only present in pathogenic *C. michiganensis*. *rhuM* is only present in *C. michiganensis* tomato pathogens. Primers used to amplify each gene are shown in gray. **B**, Agarose gel electrophoresis of multiplex PCR assay products. Total genomic DNA was used as a template with the RhuM-F/R primers targeting the *rhuM* gene, the TomA-F1/R1 primers targeting the *tomA* gene, and the 16S-R-F/R primers targeting 16S ribosomal RNA (rRNA) in either bacteria or plants. Lanes 1 to 5, endophytic *Clavibacter* strains (CASJ009, CFBP8017, CFBP8019, CFBP7576, and CFBP7494); lanes 6 to 39, pathogenic *C. michiganensis* (CFBP7311, CFBP1940, CFBP5842, CFBP7568, CFBP7488, CFBP2500, CFBP7158, CFBP7312, CFBP7316, CFBP7314, CFBP6885, CFBP7315, CFBP7589, NCPPB382, ATCC14456, ATCC10206, NZ2541, NZ1811, NZ5026, CASJ001, CASJ002, CASJ003, CASJ004, CASJ005, CASJ008, CA00001, CAYO001, AZ-28, NZ2550, 285D, 12B, 13C1, Cm4, and Cm5); lanes 40 to 43, *C. sepedonicus* (NZ2535, SB109, INM-1, and BRR7); lane 44, *C. insidiosus* (CIC266); lanes 45 and 46, *C. tessellarius* (CIC021 and CIC022); lanes 47 and 48, *Xanthomonas euvesicatoria* (RL677 and HB1); lanes 49 to 51 *Pseudomonas syringae* pv. *tomato* (PstA9, Pst18, and Pst838-8); lanes 52 to 54, *Escherichia coli*, 'Candidatus Liberibacter asiaticus' HHCA, and uninfected tomato DNA; and lane M = 1-kb DNA ladder.

and 14 days postinoculation from 1, 2, 3, 6, 9, and 15 cm above the inoculation site, including from leaves. Samples were ground and split in two for DNA extraction and determining bacterial titers (Fig. 4). *C. michiganensis* was detected from all stem and leaf tissue

samples of inoculated plants at both time points, whereas it was not detected from mock-inoculated controls (Fig. 4). Under our experimental conditions, wilting disease symptoms were visible at 5 to 7 days postinoculation. For infected samples, the expected

Fig. 4. Detection of *Clavibacter michiganensis* in infected plant materials. **A**, Three-week-old tomato plants (*Solanum lycopersicum* 'Early Pak 7') were stabbed with a needle and inoculated with 5 μ l of a suspension of CASJ002 at 10^7 CFU/ml. The diagram indicates points of sample collection above the *C. michiganensis* inoculation site. **B**, i and ii, Detection of *C. michiganensis* by multiplex PCR and bacterial titers in inoculated tomato plants 7 days postinoculation (dpi). DNA was extracted from 100 mg of plant tissue with the cetyltrimethylammonium bromide (CTAB) method, resuspended in 50 μ l of water, and 2 μ l of DNA was used per multiplex PCR. **C**, i and ii, Detection of *C. michiganensis* by multiplex PCR and bacterial titers in CASJ002-inoculated tomato plants at 14 dpi. Bacterial titers, DNA extraction, and multiplex PCR were performed as described in **B**. **D**, Detection limit for CASJ002 after CTAB DNA extraction from tomato stems. DNA was extracted from plant samples 9 cm above the site of inoculation at 7 dpi. DNA samples were diluted and the total DNA concentration in each multiplex PCR is indicated above each lane. **E**, Detection of *C. michiganensis* by multiplex PCR in serial dilutions of CASJ002 DNA (50 ng) and healthy tomato plant DNA (50 ng) mixed in different ratios using 100 ng/ μ l in a 25- μ l PCR. Numbers indicate bacterial/plant DNA ratios. Lane M = size marker (1-kb DNA ladder).

amplicons of 1,000 bp (*rhuM*), 630 bp (*tomA*), and 415 bp (16S rRNA) were observed, whereas only the 415-bp fragment was observed for DNA extracted from mock-inoculated plants (Fig. 4Bi and Ci). We did not collect samples at 15 cm above the inoculation site at 7 days postinoculation because the plants were too small in height at this time point. However, the mPCR assay was able to detect *C. michiganensis* up to 9 cm above the inoculation site at 7 days postinoculation (Fig. 4Bi). The mPCR assay was able to detect *C. michiganensis* up to 15 cm above the site of infection at 14 days postinoculation, which corresponded to 8 log CFU/mg of plant tissue (Fig. 4C ii).

The limit of detection of the mPCR assay in plant samples was also determined. DNA was isolated from tomato plants with the CTAB method (cultivar EP-7). The bacterial titers were experimentally determined as previously described and used to calculate the limit of detection (Fig. 4D). The level of sensitivity for in planta detection was similar to that obtained with the bacterial cultures, and we were able to detect *C. michiganensis* in 0.02 ng of plant DNA, corresponding to approximately 10^3 *C. michiganensis* cells per PCR (Fig. 4D). The sensitivity of the mPCR was also evaluated by spiking *C. michiganensis* CASJ002 DNA into uninfected tomato DNA samples in bacterial/tomato DNA ratios 1:1, 1:5, 1:25, 1:125, 1:250, and 1:312.5 with dilution of 100 ng/μl in each PCR. In these experiments, *C. michiganensis* was detected down to a *C. michiganensis*/tomato DNA dilution of 1:250 (Fig. 4E).

We also tested the mPCR assay for detection of *C. michiganensis* in DNA extracted from artificially infested seed. Different concentrations of *C. michiganensis* strain CASJ002 were used to infest tomato seed, followed by DNA extraction and mPCR (Fig. 5A). The mPCR assay was able to detect *C. michiganensis* with bacterial contamination in DNA isolated from a sample of 10 infested seeds (Fig. 5A). Next, we evaluated the mPCR for detecting *C. michiganensis* contamination after spiking contaminated seed into clean seed lots. Seed infested with 5×10^7 *C. michiganensis* CASJ002 were mixed with 10,000 clean seeds in different ratios followed by DNA extraction and mPCR. The mPCR was able to detect 0.3% incidence of *C. michiganensis* contamination in seed (Fig. 5B).

Evaluation of mPCR detection from recent *C. michiganensis* field strains. In 2016, there were bacterial canker occurrences in the California Central Valley (Supplementary Table S3). Tomato samples exhibiting symptoms of wilt, canker, and bird's eye spots on fruit were collected from fields and assayed for the presence of *C. michiganensis* (Supplementary Table S3; Supplementary Fig. S4). The three fragments—*rhuM* (1,000 bp), *tomA* (630 bp), and 16S rRNA (415 bp)—were amplified from samples with canker symptoms, whereas only the fragment corresponding to 16S rRNA was amplified from healthy plants (Fig. 6A). With the mPCR assay, *C. michiganensis* was detected in all 21 samples with canker symptoms tested, consistent with validation of *C. michiganensis* from the samples on D2 medium. Furthermore, we verified the pathogenicity of selected *C. michiganensis*-like colonies by inoculating tomato EP-7. Samples that tested positive using the mPCR assay generated *C. michiganensis*-like colonies on D2 media. These colonies also caused bacterial canker disease symptoms and grew to levels similar to those of previously characterized strains, including CASJ002 (Fig. 6B and C). Taken together, these data demonstrate the specificity and utility of mPCR detection of *C. michiganensis* in field samples and diagnosis of bacterial canker.

DISCUSSION

In this study, we used a comparative genomics approach to analyze strains and species of the genus *Clavibacter* and used this information to develop a specific detection assay for *C. michiganensis*, the causal agent of bacterial canker of tomato. Here, we have identified orthologous loci that are distributed among

Clavibacter spp. In total, 1,258 core orthologous loci were used to generate high-resolution *Clavibacter* phylogeny, which clearly demonstrates that *C. michiganensis* is separate from other *Clavibacter* spp. The highly similar orthologous loci among the *C. michiganensis* strains also indicates their conserved function. The analyses of the *C. michiganensis* genomes supports the monophyletic nature of this pathogen. However, *C. michiganensis* strains exhibit genetic diversity when compared with each other. The *chp/tomA* pathogenicity island is conserved among all the *C. michiganensis* strains and has been previously demonstrated to be a critical virulence factor in *C. michiganensis* strain NCPPB382 (Gartemann et al. 2008; Kaup et al. 2005). Significant differences in plasmid content and composition were observed, consistent with previous findings of plasmid diversity in *C. michiganensis* strains from California and New York (Thapa et al. 2017). Our previous study on California *C. michiganensis* strains also had similar findings (Thapa et al. 2017). Differences in genomic island, secreted protein, and CAZymes were also observed (Tancos et al. 2015; Thapa et al. 2017).

The *C. michiganensis* strains in our present phylogeny do not cluster based on geographical origin or the year of isolation, which is consistent with seedborne disease and separate introduction events. A similar distribution pattern was observed for *C. michiganensis* outbreaks in Turkey (Baysal et al. 2011). This is also supported by studies reporting a single source of introduction by seed which persisted afterward in the region (de León et al. 2009; Jacques et al. 2012; Kawaguchi et al. 2010). Our comparative genomics approach further identified loci that are conserved in all *C. michiganensis* strains and absent in other *Clavibacter* spp. and bacterial pathogens of tomato. Of 10 loci examined, 2 were used to design a *C. michiganensis* detection assay based on mPCR. We demonstrated the power of a genomics approach for comparing a

Fig. 5. Detection of *Clavibacter michiganensis* in seed. **A**, *C. michiganensis* multiplex PCR detection from contaminated tomato seed. Tomato seeds were contaminated with CASJ002 at the indicated concentrations. DNA was directly extracted from 10 seeds, the pellet was resuspended in 50 μl, and 2 μl of purified DNA was used in each multiplex PCR. **B**, Detection of *C. michiganensis* by multiplex PCR after spiking contaminated seed with clean seed lots consisting of 10,000 seeds. Seed infested with 5×10^7 CASJ002 were mixed with clean seed in different ratios followed by DNA extraction and multiplex PCR. Seed were incubated with 120 ml of phosphate-buffered saline and crushed in a stomacher, and 5 to 10 ml was used for DNA extraction. The pellet was resuspended in 100 to 200 μl and 2 to 4 μl of purified DNA was used in each PCR. Infested: clean seed ratios are indicated above each lane. Lane 1 = 1-kb DNA ladder.

large number of related bacterial genomes to identify specific genetic markers for pathogen detection.

The best way to manage bacterial canker disease of tomato is using a seed source free of *C. michiganensis* contaminants, extensive sanitation of equipment, removal of crop debris, and crop rotation in the field. Effective detection methods are critical for early detection of outbreaks and for detection of contaminated seed lots. Even low levels of *C. michiganensis* contamination (0.01%) from seed to seedling can cause a disease epidemic under favorable conditions (Chang et al. 1991). Therefore, accurate detection of *C. michiganensis* in seed is an important component of disease management, especially given the prevalence of diverse endophytic *Clavibacter* strains associated with tomato. The most common detection assays for *C. michiganensis* are serological or PCR based, following isolation of suspect bacteria by growth on semiselective media (Alvarez et al. 2005; Dreier et al. 1995; ISHI 2017; Louws et al. 1998; Kaneshiro et al. 2006; Milijašević-Marčić et al. 2012). The serological techniques are not reliable due to their cross reaction with other *Clavibacter* spp. (Dreier et al. 1995; Franken et al. 1993; Jacques et al. 2012). Most of the present PCR assays for *C. michiganensis* are based on plasmid targets (Alvarez et al. 2005; Dreier et al. 1995; Kleitman et al. 2008; Santos et al. 1997). Whereas all pathogenic *C. michiganensis* strains possess at least one virulence plasmid, we have identified significant variation in *C. michiganensis* plasmid content and profiles (Thapa et al. 2017). For example, the NZ2541 strain lacks pCM1 but is able to cause disease on tomato. Furthermore, only 85% of the *C. michiganensis*

strains sequenced possess pCM2-like plasmids. Other studies have reported *C. michiganensis* plasmid variation in field strains and plasmid loss under laboratory conditions (Kleitman et al. 2008; Tancos et al. 2015; Thapa et al. 2017). For example, the frequently used CM5/CM6 primer pair, which targets the *pat-1* locus in pCM2, only directed the amplification of the target fragment from 75% of tested *C. michiganensis* strains, whereas the CM3/CM4 primer pair, which targets the *ppaJ* locus in pCM1, directed the amplification of the target fragment from 83% of tested *C. michiganensis* strains (Hadas et al. 2005; Louws et al. 1999; Santos et al. 1997). In the present study, these primers showed both false-positive and -negative results (Supplementary Table S2). Therefore, the published primers targeting plasmids for detection of *C. michiganensis* are not sufficiently reliable.

Diagnostic tests for pathogen presence should possess specificity, sensitivity, and reliability. In our mPCR diagnostic assay for *C. michiganensis*, we included three primer pairs that target two *C. michiganensis* chromosomal loci (*tomA* and *rhuM*), as well as the 16S rRNA gene as an internal control that amplifies bacterial or plant mitochondrial DNA. *tomA* is found in the *chp/tomA* pathogenicity island, which is present in all pathogenic *C. michiganensis* strains but is absent in *Clavibacter* endophytes and *Clavibacter* spp. infecting other crops (Kaup et al. 2005; Thapa et al. 2017). *tomA* encodes a putative tomatinase, which deglycosylates the antibacterial saponin α -tomatine (Kaup et al. 2005). The *rhuM* gene is also conserved in all *C. michiganensis* strains (Supplementary Table S1). *rhuM* is predicted to be

Fig. 6. Multiplex PCR confirmed the presence of *Clavibacter michiganensis* in samples collected from commercial production fields. **A**, Detection of *C. michiganensis* by multiplex from field samples. Purified *Clavibacter*-like bacterial colonies were used for DNA extraction. Lane 1, positive control containing DNA from CASJ002; lanes 2 to 22, samples collected from different fields in California; and lane 23, tomato DNA. The *RhuM*-F/R primer pair targets the *rhuM* gene, the *TomA*-F1/R1 primer pair targets the *tomA* gene, and the 16S-R-F/R primer pair targets the 16S ribosomal RNA (rRNA) gene. Lane M = 1-kb DNA ladder (NEB). **B**, Bacterial wilt symptoms induced by *C. michiganensis* strains CASJ002, CA16_1068, CA16_1071, CA16_1073, CA16_1083, CA16_1085, and CA16_1094 in tomato 14 days postinoculation (dpi). Three-week-old tomato plants (*Solanum lycopersicum* 'Early Pak 7') were stabbed with a needle and inoculated with a 5- μ l bacterial suspension of 10^7 CFU/ml. **C**, Bacterial titers in infected tomato plant materials reached levels similar to that of CASJ002 14 dpi. Three-week-old tomato plants (*S. lycopersicum* 'Early Pak 7') were stabbed with a needle and inoculated with a 5- μ l bacterial suspension of 10^7 CFU/ml; n = 6 plants per strain and error bars = standard deviation.

involved in virulence in other bacterial pathogens based on its presence on the PAI-3 of *Salmonella* spp. but its function in *C. michiganensis* virulence remains to be determined (Bertelloni et al. 2017). We verified the specificity of the mPCR assay against 75 *C. michiganensis* strains, 20 other *Clavibacter* strains, and 15 other bacterial plant pathogens. We detected *C. michiganensis* at as little as approximately 10^3 CFU/ml, which is a level of sensitivity similar to that also reported for PCR in other studies (Dreier et al. 1995; Luo et al. 2008). We were also able to detect *C. michiganensis* from seed after artificial contamination. The mPCR assay reported herein proved to be a sensitive, accurate, and reliable diagnostic technique for *C. michiganensis* in infected plant materials.

Recent advancements in genome sequencing and high-throughput computing have enabled pathogen tracking and development of pathogen detection assays (Bueno-Sancho et al. 2017; Islam et al. 2016; Langlois et al. 2017; Savory et al. 2017). For instance, comparative analysis has been used to identify emergence of the *Magnaporthe oryzae* lineage and its origin in Bangladesh, and track and define *Puccinia striiformis* population structure and dispersal on a global scale (Bueno-Sancho et al. 2017; Islam et al. 2016). It has also been used for real-time monitoring of *Phytophthora infestans* populations for virulence and application of preventive fungicide (Li et al. 2012). Comparative genomics has also enabled specific detection and identification of different plant-pathogenic bacteria (Langlois et al. 2017; Savory et al. 2017). Our results demonstrate the successful application of comparative genomics to identify specific loci for accurate detection of *C. michiganensis*. The genetic markers obtained from whole-genome comparative analysis used in the mPCR assay described here are specific, sensitive, and reliable for the detection of *C. michiganensis* in both infected tomato plants and seed. The adoption of this assay by the seed industry and diagnostic laboratories will facilitate rapid screening of infected plants and seed for routine diagnostics.

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