Loop-Mediated Amplification of the *Clavibacter michiganensis* subsp. *michiganensis* *micA* Gene Is Highly Specific

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ABSTRACT

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Loop-mediated amplification (LAMP) was used to specifically identify *Clavibacter michiganensis* subsp. *michiganensis*, causal agent of bacterial canker of tomato. LAMP primers were developed to detect *micA*, a chromosomally stable gene that encodes a type II lantibiotic, michiganin A, which inhibits growth of other *C. michiganensis* subspecies. In all, 409 bacterial strains (351 *C. michiganensis* subsp. *michiganensis* and 58 non-*C. michiganensis* subsp. *michiganensis*) from a worldwide collection were tested with LAMP to determine its specificity. LAMP results were

compared with genetic profiles established using polymerase chain reaction (PCR) amplification of seven genes (*dnaA*, *ppaJ*, *pat-1*, *chpC*, *tomA*, *ppaA*, and *ppaC*). *C. michiganensis* subsp. *michiganensis* strains produced eight distinct profiles. The LAMP reaction identified all *C. michiganensis* subsp. *michiganensis* strains and discriminated them from other *C. michiganensis* subspecies and non-*Clavibacter* bacteria. LAMP has advantages over immunodiagnostic and other molecular detection methods because of its specificity and isothermal nature, which allows for easy field application. The LAMP reaction is also not affected by as many inhibitors as PCR. This diagnostic tool has potential to provide an easy, one-step test for rapid identification of *C. michiganensis* subsp. *michiganensis*.

Clavibacter michiganensis subspecies *michiganensis* is the causal agent of bacterial wilt and canker of tomato (*Lycopersicon esculentum*) (4,38) and is one of the most important bacterial pathogens of tomato (7,12). This worldwide industry, valued at \$58 billion in 2011 (9), can suffer substantial economic losses from *C. michiganensis* subsp. *michiganensis* outbreaks.

Most *C. michiganensis* subsp. *michiganensis* strains harbor two circular plasmids, pCM1 (27 kb) and pCM2 (70 kb), that are involved in virulence, with varying plasmid content found among *C. michiganensis* subsp. *michiganensis* isolates (24). Dreier et al. (6) and Jahr et al. (17) showed that the plasmid-encoded pathogenicity genes *celA* (on pCM1) and *pat-1* (on pCM2) are sufficient for disease induction. The *celA* gene encodes an endo- β -1,4-glucanase, which is a 78-kDa protein (746 amino acids) belonging to the glycosyl hydrolase family 5 (cellulase family A₁) (17,31). The *pat-1* gene encodes a serine protease, which is a 29.7-kDa protein (280 amino acids) (6) having significant homology to serine proteases of the chymotrypsin type, with serine and histidine in the catalytic triad (7). The virulence plasmids can be lost by stress (e.g., growth at temperatures >30°C), resulting in a reduced-virulence phenotype, and curing of both plasmids results in total loss of virulence (31). Plasmid-free strains may attain in planta titers as high as the virulent wild-type strains but do not elicit symptoms (31), indicating that all functions required for host recognition, invasion, suppression of host defenses, and colonization of the host are encoded on the *C. michiganensis* subsp. *michiganensis* chromosome. Cured strains that maintain

one of the two plasmids, either pCM1 or pCM2, still produce a virulent phenotype; however, wilting symptoms are delayed with respect to the wild-type (31).

The *C. michiganensis* subsp. *michiganensis* chromosome includes a genomic island of $\approx$ 129 kb, which has a significantly lower GC content than that typically found in *C. michiganensis* subsp. *michiganensis* and occurs close to the origin of replication. The genomic island is flanked by two direct repeats of 1.9 kb and represents a putative pathogenicity island (PI) (11), because a deletion of this region can occur by recombination within these repeats. This PI, termed the *chp/tomA* region, is subdivided into subregions based on structural features (7). Proteins encoded by the *chp* subregion of the chromosome share sequence homology with the Pat-1 protein and were designated *chpA-G* (chromosomal homology to *pat-1*). These genes encode proteases belonging to serine protease family S1A and display the same general structure (3,37). Inactivation of *chpC*, as well as *ppaA* and *ppaC*, leads to a reduction in colonization titer, which results in a reduced virulence phenotype (11,37). The *tomA* subregion carries the *tomA* gene that encodes for tomatinase, which deglycosylates the antifungal and antibacterial saponin α -tomatine (23). TomA of *C. michiganensis* subsp. *michiganensis* is specific for α -tomatine and cleaves off the tetrasaccharide β -lycotetraose. The *tomA* mutants of *C. michiganensis* subsp. *michiganensis* show higher sensitivity to α -tomatine than the wild type in plate assays; however, *tomA* mutants show no reduction in virulence (23). Perhaps in wild species of tomato or in cultivars with high concentrations of tomatine, *tomA* may have a protective effect, in that detoxification of tomatine may suppress plant defense mechanisms.

Avirulent mutants (cured derivatives lacking pCM1 and pCM2) are able to colonize the tomato plant without producing symptoms, and reacquisition of the plasmids restores virulence (7,12). Thus, it is possible that loss of plasmids in a natural *C. michiganensis* subsp. *michiganensis* population during high tempera-

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tures associated with tomato harvest results in frequent isolation of avirulent strains from tomato stems and seed. Whether or not these populations can be complemented in the infection court or restored to virulence by the reacquisition of plasmids from other *C. michiganensis* subsp. *michiganensis* cells carrying the plasmids is unknown. There is little information on the epidemiology and the population structure of *C. michiganensis* subsp. *michiganensis* in areas where outbreaks are common (7), and the role of avirulent strains during infections is inconclusive (20).

Contaminated seed is the major inoculum source leading to outbreaks of bacterial canker in agriculture (12). As a result, *C. michiganensis* subsp. *michiganensis* is classified as a quarantine organism in many countries, with cuttings and seed of tomato requiring certification as *C. michiganensis* subsp. *michiganensis* free. The European Plant Protection Organization has released minimal standards for the identification of *C. michiganensis* subsp. *michiganensis* in plants and seed (8). Immunodiagnostic and molecular methods based on primers developed from ribosomal RNA genes, repetitive sequences, or known virulence genes are available to confirm identity of *C. michiganensis* subsp. *michiganensis*, following isolation of suspect bacteria by growth on semiselective media (5,29,30,33,36). Pathogenicity tests are time consuming and unreliable because avirulent populations occur frequently in natural *C. michiganensis* subsp. *michiganensis* populations. Additionally, polymerase chain reaction (PCR) assays based solely on virulence genes may produce false results (1,2,20,24). In this article we evaluate an alternative diagnostic method for a genetically diverse population of *C. michiganensis* subsp. *michiganensis* strains from an international collection. Loop-mediated amplification (LAMP) was utilized as the diagnostic tool for this study, with primers being designed to detect a single gene, *micA*, in order to specifically detect all *C. michiganensis* subsp. *michiganensis* strains, both virulent and avirulent.

MATERIALS AND METHODS

Bacterial strains and culture conditions. In total, 409 bacterial strains were tested in this study. Strains with K, A, and C numbers (numbers 100, 11, and 23, respectively) were from the Pacific Bacterial Collection at the University of Hawai'i at Mānoa and determined to be *C. michiganensis* subsp. *michiganensis*; A and C strains were previously characterized by bacteriological, genetic, and pathogenicity tests (22) and K strains were also characterized by *dnaA* sequence analysis (35). Another set of 233 strains, designated by T numbers, were acquired from OmniLytics Inc. (Salt Lake City, UT) and subcultured. Strains T001 to T016 were positive with *C. michiganensis* subsp. *michiganensis*-specific immunostrips (OmniLytics Inc., unpublished report) but did not have the cultural morphology of *C. michiganensis* subsp. *michiganensis*. When analyzed by 16S PCR, they had closest identity with *Ochrobactrum* spp. (T001, T002, and T013 to T015) and *Microbacterium* spp. (T003 to T012 and T016). All other T strains were identified as *C. michiganensis* subsp. *michiganensis* by phenotypic tests. In total, 42 additional non-*C. michiganensis* subsp. *michiganensis* strains were tested: 2 *C. michiganensis* subsp. *sepedonicus* (K090 and *C. michiganensis* subsp. *sepedoni-*

cus from the Netherlands), 2 *C. michiganensis* subsp. *insidiosus* (K091 and *C. michiganensis* subsp. *insidiosus* from the Netherlands), *C. michiganensis* subsp. *nebraskensis* (from the Netherlands), *C. michiganensis* subsp. *tessellarius* (from the Netherlands), an *Actinobacter* sp., *Agromyces* sp., *Burkholderia* sp., *Cellulosimicrobium* sp., *Enterobacter* sp., *Micrococcus* sp., *Pectobacterium* sp., 5 *Ralstonia* spp., 3 *Staphylococcus* spp., 17 *Clavibacter* spp. from tomato seed, and 2 additional *Microbacterium* spp. Strains were removed from -80°C , plated onto 2,3,5-triphenyl-tetrazolium chloride (TZC)–sucrose medium (agar at 17 g/liter, peptone at 10 g/liter, sucrose at 5 g/liter, and 0.001% TZC), and then incubated at 26°C ($\pm 2^{\circ}\text{C}$). Isolated colonies were streaked onto YSC medium (agar at 17 g/liter, yeast extract at 10 g/liter, sucrose at 20 g/liter, and CaCO_3 at 20 g/liter) and incubated at 26°C ($\pm 2^{\circ}\text{C}$) for large-quantity production. Bacterial cells were harvested from YSC plates (≈ 50 to $250\ \mu\text{l}$) and put into 1.5-ml microfuge tubes for DNA extraction or storage at -20°C for later use, or added to cryotubes containing Luria broth (tryptone at 10 g/liter, NaCl at 2.5 g/liter, and yeast extract at 5 g/liter) with 25% glycerol for storage at -80°C as subcultures. For pathogenicity testing, strains were removed from -80°C , plated onto peptone-sucrose-agar medium (agar at 17 g/liter, peptone at 10 g/liter, and sucrose at 5 g/liter) and then incubated at 26°C ($\pm 2^{\circ}\text{C}$).

DNA extraction. A Chelex DNA extraction was performed on samples contained in the 1.5-ml microfuge tubes. Briefly, 0.75 to 1.0 ml of 40% Chelex 100 resin (Bio-Rad, Hercules, CA) in 1× Tris-EDTA buffer (10 mM Tris HCl and 1 mM EDTA, pH 8) with 10% Triton X-100 (Sigma-Aldrich, St. Louis) was added to each tube. Samples were mixed with vigorous pipeting and vortexing and then heated to 95°C for 10 min on a digital heat block. Samples were stored at 4°C for at least 24 h to allow for separation of the DNA.

LAMP. A LAMP diagnostic reaction was developed for *C. michiganensis* subsp. *michiganensis* using the LAMP primer design program (PrimerExplorer) located on the Eiken Chemical Co., Ltd. (Tokyo) website (<http://primerexplorer.jp/e/>). Primers were designed to amplify the *micA* (*michiganin A*) region of the *C. michiganensis* subsp. *michiganensis* genome (Table 1). LAMP reactions were performed in triplicate and contained 5 μl of bacterial DNA ($\approx 5\ \text{pg}$) and 20 μl of LAMP reaction master mix: *micA*LAMP2-F3 (0.2 μM), *micA*LAMP2-B3 (0.2 μM), *micA*LAMP2-FIP (1.6 μM), *micA*LAMP2-BIP (1.6 μM), *micA*LAMP2-LoopF (0.8 μM), *micA*LAMP2-LoopF Probe (0.08 μM), Quencher probe (0.16 μM), dNTP mix (1.2 mM), 10× ThermoPol Reaction Buffer (New England Biolabs, Ipswich, MA) (2 mM), betaine (1 M), MgSO_4 (4 mM), *Bacillus stearothermophilus* DNA polymerase (New England Biolabs) (8 U), and double-distilled (dd) H_2O . Negative controls contained 5 μl of dd H_2O and 20 μl of LAMP reaction master mix. LAMP reactions were run and analyzed using the iQ5 Multicolor Real-Time PCR Detection System (Bio-Rad) under the following conditions: 65°C for 1 h, with fluorescence readings being taken at 1-min intervals, and then a final 5 min at 85°C .

PCR. PCR amplifications were carried out using six primer pairs designed to amplify six different regions of the *C. michi-*

TABLE 1. Loop-mediated amplification (LAMP) primers used in this study

Oligonucleotide primer	Sequence (5'–3') ^a	Source, reference
<i>micA</i> LAMP2-F3	CGACAACAGGAACACAGGT	This study
<i>micA</i> LAMP2-B3	GCCACATTCGATGGTGAGC	This study
<i>micA</i> LAMP2-FIP	GAGCAGCATGTCCCACCGGGACACGATGAACGACATCCTC	This study
<i>micA</i> LAMP2-BIP	CGTCCGTCCAGACCCAGATCGCTGGACATGTACGGGCTCA	This study
<i>micA</i> LAMP2-LoopF	TGACCATGACGGGGGTCT	This study
<i>micA</i> LAMP2-LoopF probe	/56-FAM/ACGCTGAGGACCCGGATGCGAATGCGGATGCGGATGCCGATGACCATGACGGGGGTCT	This study
Quencher probe	TCGGCATCCGCATCCGCATTCGCATCCGGTCTCTACGCGT/3BHQ_1/	26

^a /56-FAM/ = 5'-carboxyfluorescein and /3BHQ_1/ = 3' black hole quencher 1.

ganensis subsp. *michiganensis* genome (one region on each plasmid and four chromosomal regions). Two of the PCR primer pairs are commercially available and were designed to detect pathogenicity plasmids pCM1 (CM₃/CM₄; *ppaJ*) (36) and pCM2 (Cmm5/Cmm6; *pat-1*) (5). Four primers were designed to amplify the *tomA* (tomatinase A) and *chpC* (chromosomal homology of *pat-1*), along with pathogenicity genes *ppaA* and *ppaC*, contained within the putative PI of the chromosome. Primers used to detect the *dnaA* gene were replication initiation factor primers developed by Schneider et al. (35). The primer sequences are listed in Table 2. The primer sequence used for Cmm5 was developed by Dreier et al. (5), with a 1-nucleotide (nt) change by Kokošková et al. (25). PCR reactions for all primers except *dnaA*/R were performed in a 25- μ l reaction volume containing 5 μ l of bacterial DNA and 20 μ l of PCR reaction master mix: primers (0.2 μ M each), 5 $\times$ Green GoTaq Reaction Buffer (Promega Corp., Madison, WI) (2 mM), dNTP mix (0.2 mM), MangoTaq DNA Polymerase (Bioline, Randolph, MA) (1 U), and ddH₂O. For *dnaA*/R, reactions were performed in a 25- μ l reaction volume containing 5 μ l of bacterial DNA and 20 μ l of PCR reaction master mix: primers (1 μ M each), 5 $\times$ Green GoTaq Reaction Buffer (Promega Corp.) (2 mM), dNTP mix (0.2 mM), MangoTaq DNA Polymerase (Bioline) (1 U), and ddH₂O. PCR reaction conditions for all primers used can be found in Table 2. PCR products were resolved using 1.5% agarose gel electrophoresis. Gels were analyzed using the Foto/Analyst Express System (Fotodyne Inc., Hartland, WI).

DNA sequencing. PCR products for *dnaA*/R were purified using a MinElute PCR Purification Kit (Qiagen, Valencia, CA), and the products were sequenced at the University of Hawaii sequencing facilities, using the same forward and reverse primers, according to their specifications.

Phylogenetic analyses. Sequence alignment and phylogenetic analyses were performed using MEGA5 version 5.05 (39). Sequence data for *C. michiganensis* subsp. *michiganensis* strains NCPPB 382, K0074, K0079, K0084, K0087, K0428, K0448, K0449, K0465, and K0469 (accession numbers AM711867.1, HM181168.1, HM181170.1, HM181247.1, HM181274.1, HM181201.1, HM181213.1, HM181216.1, HM181227.1, and HM181230.1, respectively); *C. michiganensis* subsp. *insidiosus* strain K0091 (accession number HM469685.1); *C. michiganensis* subsp. *sepedonicus* strain K0090 (accession number HM181287.1); and out-group *Aquifex aeolicus* (accession number AE000657.1) were obtained from the National Center for Biotechnology Information GenBank. Sequences were trimmed to 661 nt and multiple alignments were made using ClustalW, taking into account the corresponding amino acid alignments for protein-coding genes. Maximum-parsimony analysis was used to construct trees, with the closest neighbor interchange at search level 3 (32).

Neighbor-joining (NJ) analyses (34) were constructed using the Jukes-Cantor method (19) to compute evolutionary distances. Confidence intervals were assessed using the bootstrap method with 1,000 replications (10).

Nucleotide accession numbers. The *Clavibacter* spp. partial *dnaA* sequences have been deposited into the GenBank Database with accession numbers KC416011 to KC416025.

Pathogenicity testing. A sterile scalpel was used to make a vertical incision ($\approx$ 1 mm) on the stem, between the cotyledons, of juvenile tomato plants (*L. esculentum* 'Kewalo') that were $\approx$ 3 to 4 weeks old. Bacteria ($\approx$ 10⁹ CFU/ml) were applied to the wound using the scalpel tip. Negative controls were made by applying 50 μ l of ddH₂O to the wound. Inoculated plants and controls were then carefully placed into plastic bags, closed using twist ties, and incubated at room temperature for 24 h. After the initial incubation, plants were removed from the bags and allowed to grow for 4 weeks in a growth room (30°C) under Philips F40/AGRO Agro-Lite fluorescent lamps (Philips, Amsterdam, NL). Plants were checked periodically for symptoms and pathogenicity was determined at 4 weeks postinoculation, relative to controls. Wilt or necrosis of true leaves or canker at the inoculation site were indicative of pathogenicity. Two plants were used per test for each strain, with the entire set of tests being repeated three times.

RESULTS

Molecular identification and bacterial characterization using LAMP and PCR. The utility of the LAMP reaction was evaluated by molecular tests performed on 409 bacterial strains that were also characterized by PCR. In all, 351 strains (85.81%) were LAMP positive and formed eight distinct genetic patterns, as determined by PCR (Table 3). Three strains (0.85%) [A4598, A4820, and A4830] were positive for LAMP but did not match any of the above profiles and were designated patterns 9 and 10 in Supplemental Table 1. All non-*C. michiganensis* subsp. *michiganensis* strains tested were negative for the LAMP reaction (Table 4). In total, 2 *Ralstonia* strains (A3292 and A3293), 5 *Ochrobactrum* strains, and 13 *Microbacterium* strains were positive for CM₃/CM₄ but negative for all other PCR tests. Interestingly, one strain of *C. michiganensis* subsp. *sepedonicus* (K90) was also positive for CM₃/CM₄. Five *C. michiganensis* strains (two *C. michiganensis* subsp. *insidiosus*, one *C. michiganensis* subsp. *nebraskensis*, and two *C. michiganensis* subsp. *sepedonicus*) were positive for *dnaA*, while the *C. michiganensis* subsp. *tessellarius* strain did not produce a reaction with the *dnaA* PCR test, suggesting that this strain may not be a true *C. michiganensis* subsp. *tessellarius*. This study included 17 *Clavibacter* strains that were isolated from tomato seed, 12 of which were from the Netherlands. These strains were subjected to the same

TABLE 2. Polymerase chain reaction primers and conditions used in this study

Primer	Sequence (5'-3')	Cycle conditions (35 cycles of)	Size (bp)	Source, reference
Cmm5 ^a	GCGAATACGCCCATATCAA	94°C for 7 min, (94°C for 1 min, 55°C for 1 min, 72°C for 1 min), 72°C for 5 min	614	5,25
Cmm6	CGTCAGGAGGTCGCTAATA	...		
CM ₃	CCTCGTGAGTGCCGGGAACGTATCC	94°C for 7 min, (94°C for 1 min, 60°C for 1 min, 72°C for 1 min), 72°C for 5 min	645	36
CM ₄	CCACGGTGGTTGATGCTCGCGAGAT	...		
chpC-F3	TCCGGAATCTCCCGGAAG	95°C for 5 min, (95°C for 15 s, 55°C for 30 s, 72°C for 30 s), 72°C for 7 min	203	This study
chpC-B3	CGGAGCATATATGCCCAACC	...		
tomA-F3	ATCACAGTGGTGGAAGTGCTC	95°C for 5 min, (95°C for 15 s, 55°C for 30 s, 72°C for 30 s), 72°C for 7 min	229	This study
tomA-B3	TTCAATGGCCTTTCTCCCG	...		
ppaAF	CTGGTTCTGGTTTGCGGGC	95°C for 5 min, (95°C for 30 s, 55°C for 30 s, 72°C for 30 s), 72°C for 7 min	496	This study
ppaAR	TGCTGCTGGTCTCTCTGGTA	...		
ppaCF	TGGTCGTCTGTTGAGGAGGCA	95°C for 5 min, (95°C for 30 s, 55°C for 30 s, 72°C for 30 s), 72°C for 7 min	140	This study
ppaCR	CTGTCGTCGGAGCGTCGGAT	...		
dnaAF	TACGGCTTCGACACCTTCG	94°C for 5 min, (94°C for 30 s, 61°C for 1 min, 72°C for 30 s), 72°C for 10 min	933	35
dnaAR	CGGTGATCTTCTTGTTGGCG	...		

^a Bold C indicates the change made by Kokošková et al. (25).

tests and all strains, except X-4, were positive only for *dnaA*, suggesting that they are, in fact, *Clavibacter* spp. Strain X-4 was subjected to 16S rDNA analysis and showed the closest match with *Agromyces* spp. (99% maximum ID). The remaining strains were then subjected to *dnaA* sequence analyses.

Phylogenetic analysis based on *dnaA* sequence data. The subset of nine *C. michiganensis* subsp. *michiganensis* strains used for this analysis clustered separately from six unnamed *Clavibacter* spp. isolated from tomato seed from California (17) and from eight unnamed *Clavibacter* spp. isolated from tomato seed in the Netherlands (this study) (Fig. 1). The *C. michiganensis* subsp. *michiganensis* strains also clustered separately from all other known subspecies of *C. michiganensis* (*insidiosus*, *sepedonicus*, and *nebraskensis*) (Fig. 1). NJ analyses produced consensus trees with analogous topology (data not shown).

Pathogenicity of strains representative of each distinct population group. Results of pathogenicity tests, some of which have already been reported (22), are shown in Supplemental Table 2. Strains having patterns 1, 2, 3, 4, 5 and 7 produced typical canker symptoms, mostly within 6 to 14 days, whereas strains from patterns 6, 8 produced no symptoms within the observation period (28 days after inoculation) (Table 3 and Supplemental Table 2). The three strains comprising patterns 9 and 10 as well as the *Clavibacter* spp. strains isolated from seed also were non-pathogenic on tomato.

DISCUSSION

LAMP is a relatively new molecular detection method that involves an isothermal reaction (65°C). LAMP utilizes *Bst* DNA

polymerase, which has DNA-displacement activity. At 65°C, the DNA helix is unstable, allowing the *Bst* polymerase to invade the helix and displace the two strands as it polymerizes, thus eliminating the need for denaturation. The isothermal nature of this reaction is advantageous because there is no need for expensive thermal cycling equipment, as required for PCR. LAMP has adequate sensitivity and can be highly specific through proper primer design. The LAMP reaction is also relatively fast, the with majority of reactions starting exponentially at 20 to 30 min and requiring a maximum reaction time ≤1 h. A positive LAMP reaction can be observed visually via turbidity (28), making it suitable for field application, using a noninstrumental nucleic acid amplification device (27). LAMP reactions have been adapted for visualization in real-time with fluorescent probes (26). LAMP reactions also can be used directly in the field via SMART-DART technology (18). Currently, LAMP technology is being applied to detection of several bacterial plant pathogens, including *Ralstonia solanacearum* (27,28) *Xylella fastidiosa* (13), and *Erwinia amylovora* (40,41).

In this study, a LAMP reaction was designed to amplify the *mica* region of the *C. michiganensis* subsp. *michiganensis* chromosome, which encodes an antibiotic. Many bacteria produce antibiotic compounds that are either nonribosomally synthesized antibiotics or ribosomally synthesized proteins, also termed bacteriocins. These compounds are thought to be involved in the competition among bacteria for nutrients and specific habitats. The *mica* gene encodes for a heat- and protease-resistant peptide of 2,145 Da (AMP-II), the lantibiotic michiganin A (14,15). Michiganin A is a type-II actagardine-like lantibiotic with activity against *C. michiganensis* subsp. *sepedonicus*, the potato ring-rot pathogen. *C. michiganensis* subsp. *sepedonicus* lacks the gene

TABLE 3. Representative groups of 351 *Clavibacter michiganensis* subsp. *michiganensis* strains as determined by loop-mediated amplification (LAMP), polymerase chain reaction (PCR), and pathogenicity tests^a

Strain number	Population (%)	<i>dnaA</i>	Cmm5/6	CM _{3/4}	<i>chpC</i>	<i>tomA</i>	<i>ppaA</i>	<i>ppaC</i>	LAMP	Pathogenicity	Pattern number
252	71.79	+	+	+	+	+	+	+	+	+	1
26	7.41	+	—	+	+	+	+	+	+	+	2
19	5.41	+	+	—	+	+	+	+	+	+	3
4	1.14	+	—	—	+	+	+	+	+	+	4
18	5.13	+	+	+	—	+	+	+	+	+	5
5	1.42	+	+	+	—	—	+	+	+	—	6
5	1.42	+	+	+	+	+	—	+	+	+	7
19	5.41	+	+	+	—	—	—	—	+	—	8

^a Patterns of three LAMP-positive *C. michiganensis* subsp. *michiganensis* strains (A4598, A4820, and A4830) were not identical to any of the above and are shown in Supplemental Table 1.

TABLE 4. Non-*Clavibacter michiganensis* subsp. *michiganensis* strains evaluated by loop-mediated amplification (LAMP) and polymerase chain reaction (PCR) tests

Strain number	Other designation	<i>dnaA</i>	Cmm5/6	CM _{3/4}	<i>chpC</i>	<i>tomA</i>	<i>ppaA</i>	<i>ppaC</i>	LAMP
1	<i>Acinetobacter</i> sp.	—	—	—	—	—	—	—	—
1	<i>Agromyces</i> sp.	—	—	—	—	—	—	—	—
1	<i>Burkholderia</i> sp.	—	—	—	—	—	—	—	—
1	<i>Cellulomicrobium</i> sp.	—	—	—	—	—	—	—	—
17	<i>Clavibacter</i> sp. from seed	+	—	—	—	—	—	—	—
2	<i>C. michiganensis</i> subsp. <i>insidiosus</i>	+	—	—	—	—	—	—	—
1	<i>C. michiganensis</i> subsp. <i>nebraskensis</i>	+	—	—	—	—	—	—	—
1	<i>C. michiganensis</i> subsp. <i>sepedonicus</i>	+	—	—	—	—	—	—	—
1	<i>C. michiganensis</i> subsp. <i>sepedonicus</i>	+	—	+	—	—	—	—	—
1	<i>C. michiganensis</i> subsp. <i>tessellarius</i>	—	—	—	—	—	—	—	—
2	<i>Curtobacterium</i> sp.	—	—	—	—	—	—	—	—
1	<i>Enterobacter</i> sp.	—	—	—	—	—	—	—	—
13	<i>Microbacterium</i> sp.	—	—	+	—	—	—	—	—
1	<i>Micrococcus</i> sp.	—	—	—	—	—	—	—	—
5	<i>Ochrobactrum</i> sp.	—	—	+	—	—	—	—	—
1	<i>Pectobacterium</i> sp.	—	—	—	—	—	—	—	—
1	<i>Ralstonia eutropha</i>	—	—	—	—	—	—	—	—
2	<i>R. solanacearum</i>	—	—	+	—	—	—	—	—
2	<i>R. solanacearum</i> (fliC—)	—	—	—	—	—	—	—	—
3	<i>Staphylococcus</i> sp.	—	—	—	—	—	—	—	—

cluster responsible for the production of michiganin A (7), suggesting that these compounds are utilized for population control of closely related species. This region of the *C. michiganensis* subsp. *michiganensis* genome was chosen because other genes targeted for molecular detection are either virulence factors that exist on plasmids or genes located in a PI within the chromosome. Molecular detection tests based on a single pathogenic determinant may produce false results. For example, a detection aimed at only one of the two plasmid-borne genes may give a negative result in a pathogenic strain, producing a false negative. In contrast, strains possessing both virulence plasmids could be nonpathogenic if missing the *chpC*, *tomA*, *ppaA*, and *ppaC* region. Though detected by the corresponding PCR assay, the pathogenicity test would be negative and, thus, the assay would be considered a false positive by PCR. The observation that *micA* is present on the chromosome in an area not prone to deletions suggested that this gene should be present in all *C. michiganensis* subsp. *michiganensis* strains, regardless of pathogenicity. In addition, michiganin A inhibits closely related subspecies, such as *C. michiganensis* subsp. *sepedonicus*; therefore, logically, this region would be absent from the other *Clavibacter* subspecies.

Kleitman et al. (24) found varying plasmid content among *C. michiganensis* subsp. *michiganensis* isolates. Twenty *C. michiganensis* subsp. *michiganensis* strains were tested for *celA*, *pat-1*, *chpC*, *ppaA*, and *tomA* as well as pathogenicity. Fifteen strains (75%) were pathogenic and positive for all five genes tested. The remaining five strains (25%) were nonpathogenic (24). Two of the

five nonpathogenic strains were negative only for the *chpC* region, thus confirming that the *chpC* gene of the *chp/tomA* PI alone is sufficient to produce a nonvirulent phenotype, regardless of the presence of the pathogenicity plasmids. Two other nonpathogenic strains were negative for both *pat-1* and *chpC*. The final nonpathogenic strain was only negative for *ppaA*, thus confirming this gene's role in pathogenicity. There are few known studies that include the *ppaA*, even though it has been attributed to a low colonization titer that results in reduced virulence. Additionally, no studies have been published that test for *ppaC*. In our study, we examined a large number of strains from widely separated geographical origins in order to gain a better understanding of the kinds of populations that occur in nature and to demonstrate the variety of gene content within strains that hamper current molecular detection. These results demonstrate the utility of the LAMP reaction.

In this study, all *C. michiganensis* subsp. *michiganensis* strains reacted with the LAMP whereas all non-*C. michiganensis* subsp. *michiganensis* strains failed to react, thus displaying the LAMP's potential to eliminate false positives. LAMP reactions were observed at 20 to 30 min, which is typical for a LAMP reaction (13, 18,28,40,41). Interestingly, several non-*C. michiganensis* subsp. *michiganensis* bacteria produced a positive reaction with the CM₃/CM₄ primers used to detect the presence of a gene on plasmid pCM1. These results demonstrate the potential occurrences of false positives with the commercially available primers. The majority of the *C. michiganensis* subsp. *michiganensis* strains

Fig. 1. Phylogenetic analysis of *Clavibacter* strains based on *dnaA* sequence. Maximum-parsimony (MP) tree: *dnaA* sequences were trimmed to 661 nucleotides and multiple alignments were made using ClustalW, taking into account the corresponding amino acid alignments for protein-coding genes. MP analysis was used to construct trees with the closest neighbor interchange at search level 3. Confidence intervals were assessed using the bootstrap method with 1,000 replications.

tested in this study ($n = 252$; 71.79%) were positive for all regions tested, corresponding closely to the results by Kleitman et al., who reported that 15/20 (75%) of *C. michiganensis* subsp. *michiganensis* strains were positive in PCR tests for all five genes analyzed (24). The virulence factor composition of these strains predicts that they will possess full pathogenicity. This prediction was confirmed by pathogenicity tests. Strains positive only for CM_{3/4} or Cmm5/6 are predicted to be pathogenic; however, they may have a slightly reduced or delayed pathogenicity (7,12,24). This prediction was also confirmed by the pathogenicity tests. Strains negative for both plasmid-encoded genes are predicted to be nonpathogenic. However, under the conditions of the pathogenicity tests performed here, these strains were pathogenic, which contradicts findings by other groups. Some strains were negative for only *chpC* or *ppaA*, which would suggest that these strains would be nonpathogenic. Again, these strains were pathogenic under the conditions of the pathogenicity tests performed here. It was unusual to find that strains missing the *chpC* or *ppaA* gene were pathogenic, because these results contradict the findings by other groups. A possible explanation as to why the pathogenicity tests yielded positives for patterns 5 and 7 may be the high inoculum titers. These strains possess the pathogenicity plasmids but lack the ability to colonize plants, due to the absence of key colonization genes located on the chromosome. At high inoculum levels, they may be able to produce canker symptoms without fully colonizing the plant. Further tests that allow quantification of inoculum titers for precise consistency between tests may be needed, along with testing more strains from each pattern. It is unknown why strains of patterns 4, 5, and 7 produced a pathogenic phenotype. The remaining strains were negative for both *chpC* and *tomA*, or *chpC*, *tomA*, *ppaA*, and *ppaC*, which would suggest that these strains would be nonpathogenic. These strains were found to be nonpathogenic, thus confirming previous reports. It is interesting to note that these results, along with those from Kleitman et al. (24), indicate that *chpC*, *tomA*, *ppaA*, and *ppaC*, which are all located in the same putative PI, can be lost independently of each other. Three strains were positive for LAMP but did not match any of the eight different profiles. Strains A4598 and A4820 (pattern 9) appeared to be derived from pattern 8 and strain A4830 (pattern 10) appeared to be from pattern 6; however, none of the strains reacted with Cmm5/6 and may reflect a loss of pCM2. It is possible that stress caused the curing of pCM2, whereas pCM1 remained, possibly due to its more stable nature (7,12,17).

The elevated temperatures during warm summer harvests or during the seed-drying process may stimulate the conversion of virulent strains into plasmid-free nonvirulent strains. An unforeseen cohabitation of tomato seed or plant with two nonvirulent strains, one being a plasmid-free *chpC-ppaA-ppaC*-positive strain and the other possessing the virulence plasmids but missing *chpC*, *ppaA*, or *ppaC*, is a potentially dangerous situation with possible devastating consequences. Should the nonvirulent plasmid-free strains reacquire one or both plasmids from the other nonvirulent population, restoration to full virulence would produce an outbreak. This scenario demonstrates the need for reevaluating the way these nonvirulent populations are viewed. Nonvirulent strains cannot be simply disregarded during routine seed and plant tests, and such strains need further study.

The *Clavibacter* strains isolated from tomato seed were morphologically similar to *C. michiganensis* subsp. *michiganensis* and were positive with immunostrips (2,20–22) but negative for pathogenicity. Phylogenetic analysis grouped these strains separately from *C. michiganensis* subsp. *michiganensis* and other known subspecies of *C. michiganensis*. Similar findings were recently reported by Zaluga et al. (42) and Jacques et al. (16). Such strains look like *C. michiganensis* subsp. *michiganensis* in culture and often produce false positives when using current immunodiagnostic methods; however, the LAMP assay described

here was able to distinguish such strains from true *C. michiganensis* subsp. *michiganensis*. This study validates the use of the *micA* gene for specific identification of *C. michiganensis* subsp. *michiganensis*, using LAMP as the diagnostic test.

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