Detection of Goss's Wilt Pathogen *Clavibacter michiganensis* subsp. *nebraskensis* in Maize by Loop-Mediated Amplification

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ABSTRACT

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The Goss's wilt pathogen, *Clavibacter michiganensis* subsp. *nebraskensis*, can cause considerable losses in maize (*Zea mays*) production. Diagnosis of Goss's wilt currently is based on symptomology and identification of *C. michiganensis* subsp. *nebraskensis*, following isolation on a semiselective medium and/or serological testing. In an effort to provide a more efficient identification method, a loop-mediated amplification (LAMP) assay was developed to detect the tripartite ATP-independent periplasmic (TRAP)-type C4-dicarboxylate transport system large permease component and tested using strains of *C. michiganensis* subsp. *nebraskensis*,

all other *C. michiganensis* subspecies and several genera of nontarget bacteria. Only strains of *C. michiganensis* subsp. *nebraskensis* reacted positively with the LAMP assay. The LAMP assay was then used to identify bacterial isolates from diseased maize. 16S rDNA and *dnaA* sequence analyses were used to confirm the identity of the maize isolates and validate assay specificity. The Cmm ImmunoStrip assay was included as a presumptive identification test of *C. michiganensis* subsp. *nebraskensis* at the species level. The Cmm-LAMP assay was further tested using symptomatic leaf tissue. The Cmm-LAMP assay was run in a hand-held real-time monitoring device (SMART-DART) and performed equally to in-lab quantitative polymerase chain reaction equipment. The Cmm-LAMP assay accurately identified *C. michiganensis* subsp. *nebraskensis* and has potential as a field test. The targeted sequence also has potential application in other molecular detection platforms.

Maize (*Zea mays* L.) is a highly versatile plant and is used as feed for animals, a cereal grain to produce food products, and ethanol for biofuels. From 2007 to 2012, maize was listed as the sixth largest crop commodity worldwide, with approximately 870 million tonnes produced in 2012, which was valued at 53 billion dollars (FAOSTAT 2012).

The Gram-positive phytopathogen *Clavibacter michiganensis* subsp. *nebraskensis* (Davis et al. 1984; Vidaver and Mandel 1974) causes Goss's vascular wilt (Schuster 1970; Wysong et al. 1973) and leaf lesions with discontinuous water-soaked spots or the appearance of freckling, resulting in blight of maize leaves (Schuster 1972; Schuster et al. 1972). Like all pathogenic *C. michiganensis* subspecies, *C. michiganensis* subsp. *nebraskensis* naturally infects the host through wounds (Eichenlaub and Gartemann 2011; Eichenlaub et al. 2006; Rocheford et al. 1985) and can survive on plant debris (Schuster and Coyne 1974; Schuster et al. 1975). *C. michiganensis* subsp. *nebraskensis* is also transmitted through seed (Biddle et al. 1985, 1990; Schuster 1972), though at very low rates. The disease was first discovered in 1969 in Nebraska (Schuster 1972; Vidaver and Mandel 1974) and has since spread throughout the United States of America and to Canada (Agarkova et al. 2011; Korus et al. 2011; Ngong-Nassah et al. 1992; Ruhl et al. 2009; Vidaver et al. 1981; Wysong et al. 1981; Wysong et al. 1973), causing varying degrees of losses from minor to severe (Calub et al. 1974a; Carlson and Wicks 1991; Doupnik and Wysong 1977; Pataky 1989; Pataky and Headrick

1988; Smidt and Vidaver 1986; Wysong et al. 1981; Wysong et al. 1973). Currently, there are no treatments labeled for maize production in the United States, chemical or otherwise, for the disease caused by *C. michiganensis* subsp. *nebraskensis*. The use of resistant/tolerant cultivars of maize is the most effective control measure (Calub et al. 1974b; Carlson and Wicks 1991; Doupnik and Wysong 1977; Ngong-Nassah et al. 1992; Pataky 1985; Pataky and Headrick 1988). Therefore, the feasible way to control Goss's wilt disease is hybrid selection for disease resistance, combined with crop rotation, tillage to bury infected residue, and phytosanitary inspections of seed production to assure pathogen-free seed.

Diagnosis of the Goss's wilt pathogen is based on observed symptoms and identification of *C. michiganensis* subsp. *nebraskensis* after isolation on a semiselective medium (Gross and Vidaver 1979) or direct serological assay of symptomatic leaf tissue (Korus 2011). However, disease symptoms often appear similar to Stewart's wilt (Pataky 1985, 1989; Pataky and Headrick 1988), which is caused by *Pantoea stewartii*, and CNS medium (Gross and Vidaver 1979) does permit the growth of other *C. michiganensis* subspecies and non-*Clavibacter* species (Gross and Vidaver 1979; Yasuhara-Bell 2014; Yasuhara-Bell and Alvarez 2015), making bacterial colony identification difficult. Biochemical, immunological and molecular-based assays have been used to identify *C. michiganensis* subsp. *nebraskensis*. The Biolog Microbial ID System (Biolog Inc., Hayward, CA) can identify bacteria based on carbon source utilization and chemical sensitivity; however, the Biolog database, which is compared with the biochemical signature of the test bacterium to provide proper identification, is not adequate at this time for *C. michiganensis* subspecies differentiation (Harris-Baldwin and Gudmestad 1996; Kaneshiro 2003; Kaneshiro et al. 2006; Korus 2011; Yasuhara-Bell 2014; Yasuhara-Bell and Alvarez 2015). Several immunoassays are

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available for detecting *Clavibacter michiganensis* subsp. *michiganensis*, which causes bacterial canker and wilt of tomato, including enzyme-linked immunoassays (ELISA), immunofluorescence assays, and a portable ImmunoStrip assay (Agdia Inc., Elkhart, IN). As common antigens are shared among all *C. michiganensis* subspecies, these assays also can be used for presumptive detection of *C. michiganensis* subsp. *nebraskensis*. However, cross-reactions with non-*Clavibacter* bacteria also occur (Alvarez et al. 1993; Alvarez and Kaneshiro 1999; Alvarez et al. 2005; Gitaitis et al. 1991; Kaneshiro and Alvarez 2001; Kaneshiro et al. 2006; Kokošková et al. 2010; Krämer and Griesbach 2005; Sudarshana et al. 2012), thereby making these assays less than ideal for definitive routine identification of *C. michiganensis* subsp. *nebraskensis* (Korus 2011; Yasuhara-Bell 2014). The accuracy and usefulness of these immunoassays for the diagnosis of Goss's wilt is increased significantly when combined with the observation of typical leaf symptoms and bacterial streaming from cut leaf tissue by a trained and experienced plant disease diagnostician. Molecular-based assays for *C. michiganensis* subspecies differentiation are available (Bach et al. 2003; Louws et al. 1998; Schneider et al. 2011; Smith et al. 2001; Waleron et al. 2011; Zaluga et al. 2011), but may be impractical for many plant disease diagnostic laboratories to perform for routine sample processing. The purpose of this study was to develop a more efficient identification method for *C. michiganensis* subsp. *nebraskensis*, using loop-mediated amplification (LAMP), and test its utility for pathogen detection in field samples.

MATERIALS AND METHODS

Bacterial strains and culture conditions. Bacterial strains designated by an "A" number were from the Pacific Bacterial Collection at the University of Hawaii at Mānoa (Table 1). Bacteria were taken from -80°C , plated onto TZC-S medium (agar at 17 g/liter, peptone at 10 g/liter, sucrose at 5 g/liter, and 0.001% 2,3,5-triphenyl-tetrazolium chloride [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 ($\pm 2^{\circ}\text{C}$) and used for DNA extraction.

Plant sampling and bacteria isolation. Strains designated with a "DP" number were isolated from diseased maize during the 2014 growing season and presumptively identified based on symptomatology and serological tests (Table 2). Leaves exhibiting Goss's wilt symptoms were sampled to obtain bacterial isolates from the leaf lesions. Tissue was surface-sterilized with 70% EtOH for 30 s and blotted dry. A piece ($5 \times 10 \text{ mm}^2$) of infected tissue was excised and placed in a 1.5-ml microfuge tube with 0.85% saline solution. After 5 min the tissue was removed from the saline solution and discarded. The saline solution containing tissue exudate was lightly vortexed and streaked onto nutrient broth yeast extract (NBY) agar plates (nutrient broth at 8 g/liter, yeast extract at 2 g/liter, HK_2PO_4 at 2 g/liter, H_2KPO_4 at 0.5 g/liter, glucose at 2.5 g/liter, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ at 1 ml/liter, and agar at 15 g/liter) and then incubated for 24 h at 25°C . Bacteria were subsequently streaked onto nutrient agar and then TZC medium.

Plant samples for direct testing of symptomatic leaf tissue. Forty corn leaf samples were submitted to the DuPont Pioneer Plant Disease Diagnostic Laboratory during the 2015 growing season and diagnosed positive for *C. michiganensis* subsp. *nebraskensis* based on characteristic symptoms, presence of bacterial streaming, and positive serological assay results (Table 3). Twenty-three samples of *Xanthomonas campestris*, confirmed positive by Agdia testing (Agdia Inc.), were also included in this study, as well as four samples of diseased leaves with symptoms caused by fungal pathogens, which can easily be confused with symptoms of Goss's wilt.

Bacterial identification. Confirmatory tests were performed to positively identify bacterial isolates from diseased maize

tissues. Purified colonies were plated onto Miller-Schroth medium for *Erwinia* (MS medium) (Miller and Schroth 1972) to assess fermentative abilities. Fermenting presumptive isolates of *C. michiganensis* subsp. *nebraskensis* were considered misidentified. Nonfermenting presumptive *C. michiganensis* subsp. *nebraskensis* isolates were further characterized by serological and molecular identification methods. Nonfermenting presumptive *C. michiganensis* subsp. *nebraskensis* isolates were tested using the ImmunoStrip assay (Agdia Inc.) for *C. michiganensis* subsp. *michiganensis*, according to the manufacturer's instructions, and molecular identification was carried out by sequencing polymerase chain reaction (PCR) amplicons produced using primers designed to amplify partial 16S rDNA (Weisburg et al. 1991) gene sequences. Presumptive *C. michiganensis* subsp. *nebraskensis* isolates belonging to the genus *Clavibacter* by BLAST analysis (Altschul et al. 1990) of the 16S rDNA were further characterized by sequencing PCR amplicons produced using primers designed to amplify the *dnaA* (Schneider et al. 2011) gene sequence.

DNA extraction. Briefly, bacterial isolates were suspended in 0.75 to 1.0 ml of 20% Chelex 100 resin (Bio-Rad, Hercules, CA) in $1 \times$ TE buffer (10 mM Tris-HCl and 1 mM EDTA at pH 8) with 10% Triton X-100 (Sigma-Aldrich, St. Louis, MO). Samples were mixed with vigorous pipetting 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.

Total DNA was extracted from approximately 100 mg of putative *C. michiganensis* subsp. *michiganensis*-infected corn leaf samples using a commercial DNA extraction kit (Qiagen Gentra Puregene Yeast/Bact. Kit, Valencia, CA), according to manufacturer's instructions. The resulting DNA samples were diluted to a concentration of 0.5 ng/ μl .

DNA sequencing. PCR products were cleaned for sequencing using ExoSAP-IT (Affymetrix, Santa Clara, CA), according to the manufacturer's instructions. Cleaned PCR products were sequenced at the Greenwood Molecular Biology Facility at the University of Hawaii sequencing facility, using each forward and reverse primer, according to specifications.

Phylogenetic analyses. Sequence alignment and phylogenetic analyses were performed using MEGA5 version 5.05 (Tamura et al. 2011). The *dnaA* sequence data for *C. michiganensis* subsp. *michiganensis* strains NCPPB 382, K0074, K0079, K0428, K0448, K0449, K0465, and K0469 (accessions AM711867, HM181168, HM181170, HM181201, HM181213, HM181216, HM181227, and HM181230, respectively), *C. michiganensis* subsp. *insidiosus* strain K0091 and LMG 3663 (accessions HM469685 and JX259293, respectively), *C. michiganensis* subsp. *nebraskensis* strains 20037, 200800460, NCPPB 2579, and NCPPB 2581 (accessions KF663897 to KF663899 and HE614873, respectively), *C. michiganensis* strains from pepper PF007, PF008, PS003, PS005, and PS006 (accessions KF663902 to KF663906), *C. michiganensis* subsp. *sepedonicus* strains K0090, A2014, and ATCC33113 (accessions HM181287, KF663900, and AM849034, respectively), *C. michiganensis* subsp. *tessellarius* strain LMG 7294 (accession KF663901), *C. michiganensis* subsp. *californiensis* subsp. nov. strains C55, C59-A, C63, C6-A, C74A, and C91-A, and *C. michiganensis* subsp. *chilensis* subsp. nov. strains ZUM3064, ZUM3065, ZUM3936, ZUM4209, ZUM4210, ZUM4211, ZUM4206, and ZUM4207 (accessions KC416011 to KC416025, respectively), and out-group *Aquifex aeolicus* (accession AE000657) were obtained from NCBI GenBank. 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. Neighbor-joining (NJ) trees (Saitou and Nei 1987) were constructed using the Jukes-Cantor method (Jukes and Cantor 1969) to compute evolutionary distances. Maximum-parsimony (MP) analysis was used to construct trees with the closest neighbor interchange at search level 3 (Nei and Kumar 2000). Confidence intervals were assessed using the bootstrap method with 1,000 replications (Felsenstein 1985).

Nucleotide accession numbers. The partial 16S rDNA sequences for strains DP101, DP104, DP107B, DP108, DP109A, DP109B, DP112A, DP112B, DP113, DP114A, DP115A, DP117A, DP119A, DP121A, DP121B, DP122A, DP122B, DP134, DP136, DP137, DP139A, DP139B, DP140, DP147B, DP160, DP162A, DP162B, DP162C, DP163, DP164, DP165A, DP165B, DP165C, DP173, DP174A, and DP174B have been deposited into the GenBank Database with accession numbers KP279872 to KP279907, respectively. The partial *dnaA* sequences for DP104, DP114A, DP115A, DP117A, DP121A, DP122A, DP134, DP137, DP139B, DP164, DP165C and *C. michiganensis* subsp. *phaseoli* subsp. nov. strain LMG 27667 have been deposited into the GenBank Database with accession numbers KP279908 to KP279919, respectively.

Loop-mediated isothermal amplification. LAMP primers used in this study are shown in Table 4. The LAMP primers were developed using the LAMP primer design program (PrimerExplorer V4) located on the Eiken Chemical Co., Ltd. (Tokyo, Japan) website (<http://primerexplorer.jp/e/>) and designed to detect the tripartite ATP-independent periplasmic (TRAP)-type C4-dicarboxylate transport system large permease component (Fig. 1). LAMP reactions were performed in triplicate and contained 5 µl of sample, 5 µl of primer master mix (TRAP-F3 [0.2 µM], TRAP-B3 [0.2 µM], TRAP-FIP [1.6 µM], TRAP-BIP [1.6 µM], TRAP-Loop [0.8 µM], TRAP-Probe

[0.08 µM], and Quencher Probe [0.16 µM] [Kubota et al. 2011] per reaction) and 15 µl of Isothermal Mastermix (OptiGene, West Sussex, UK). Positive and negative controls used 5 µl of *C. michiganensis* subsp. *nebraskensis* DNA and ddH₂O and/or pathogen-free sample, respectively. LAMP reactions were run and analyzed using the iQ5 Multicolor Real-Time PCR Detection System (Bio-Rad, Hercules, CA) and a hand-held real-time assessment device (SMART-DART) (DiaGenetix Inc., Honolulu, HI) at 65°C for 30 min, with fluorescence readings taken at 1-min or 30-s intervals, respectively, and a final 2 min at 85°C. One drop of mineral oil was added to the top of each sample when using the SMART-DART to prevent evaporation. Sensitivity assessment was performed using 10-fold serial dilutions of purified *C. michiganensis* subsp. *nebraskensis* (NCPBP 2579) DNA from 1 ng/µl to 5 fg/µl. DNA was purified using the Wizard Genomic DNA purification kit (Promega Corp., Madison, WI).

RESULTS

Bacterial identification. Forty-two samples from symptomatic maize were received during the 2014 growing season with preliminary bacterial identifications of *C. michiganensis* subsp. *nebraskensis*. As many of these samples contained multiple colony types, 75 colonies were tested (Table 2). All colonies were plated on

TABLE 1. Known bacterial strains used in this study, with associated ImmunoStrip and loop-mediated amplification (LAMP) data

Strain ID	Other ID ^a	Organism ^b	ImmunoStrip	LAMP
A2431	IRRI 1427-4	<i>Acidovorax avenae</i>	–	–
A2432	BCE-12	<i>Acidovorax avenae</i>	–	–
A2433	CHY9NF	<i>Acidovorax avenae</i>	–	–
A3608	ATCC 33619	<i>Acidovorax cattleyae</i>	–	–
A2961	C58	<i>Agrobacterium tumefaciens</i>	–	–
A3321	NCPBP 451	<i>Burkholderia andropogonis</i>	–	–
A6112	PS005; CNUPBL 1646	<i>Clavibacter michiganensis</i> from pepper	+	–
A1149	ATCC 10253	<i>C. michiganensis</i> subsp. <i>insidiosus</i>	+	–
A6134	C55; CFBP 8216 ^T	<i>C. michiganensis</i> subsp. <i>californiensis</i> subsp. nov.	+	–
A6101	ZUM3936; CFBP 8217 ^T	<i>C. michiganensis</i> subsp. <i>chilensis</i> subsp. nov.	+	–
A2058	H160	<i>C. michiganensis</i> subsp. <i>michiganensis</i>	+	–
A6094	NCPBP 2579; LMG 3698	<i>C. michiganensis</i> subsp. <i>nebraskensis</i>	+	+
A6095	20037	<i>C. michiganensis</i> subsp. <i>nebraskensis</i>	+	+
A6096	200800460	<i>C. michiganensis</i> subsp. <i>nebraskensis</i>	+	+
A6135	LPPA 982 ^T ; LMG 27667 ^T	<i>C. michiganensis</i> subsp. <i>phaseoli</i> subsp. nov.	+	–
A2041	R8	<i>C. michiganensis</i> subsp. <i>sepedonicus</i>	+	–
A6109	LMG 7294; ATCC 33566	<i>C. michiganensis</i> subsp. <i>tessellarius</i>	+	–
A5415	CFBP 2048	<i>Dickeya chrysanthemi</i> biovar <i>chrysanthemi</i>	–	–
A6062	CFBP 3701	<i>D. chrysanthemi</i> biovar <i>chrysanthemi</i>	–	–
A6063	CFBP 3262	<i>D. chrysanthemi</i> biovar <i>chrysanthemi</i>	–	–
A5574	IPO 2117	<i>D. chrysanthemi</i> biovar <i>parthenii</i>	–	–
A5641	CFBP 1270	<i>D. chrysanthemi</i> biovar <i>parthenii</i>	–	–
A5416	CFBP 1269	<i>D. dadantii</i> subsp. <i>dadantii</i>	–	–
A5642	CFBP 3855	<i>D. dadantii</i> subsp. <i>dadantii</i>	–	–
A5643	CFBP 6467	<i>D. dadantii</i> subsp. <i>dadantii</i>	–	–
A5419	CFBP 2051	<i>D. dadantii</i> subsp. <i>dieffenbachiae</i>	–	–
A5578	IPO 2124	<i>D. dadantii</i> subsp. <i>dieffenbachiae</i>	–	–
A5646	CFBP 3694	<i>D. dadantii</i> subsp. <i>dieffenbachiae</i>	–	–
A6060	CFBP 3698	<i>D. dadantii</i> subsp. <i>dieffenbachiae</i>	–	–
A6061	CFBP 1247	<i>D. dadantii</i> subsp. <i>dieffenbachiae</i>	–	–
A5418	CFBP 1200	<i>D. dianthicola</i>	–	–
A5571	IPO 1741	<i>D. dianthicola</i>	–	–
A5644	CFBP 2015	<i>D. dianthicola</i>	–	–
A5645	CFBP 4155	<i>D. dianthicola</i>	–	–
A6058	CFBP 1982	<i>D. dianthicola</i>	–	–
A6059	CFBP 3706	<i>D. dianthicola</i>	–	–

(continued on next page)

^a ATCC: American Type Culture Collection, USA; CFBP: Collection Française de Bactéries associées aux plantes (French Collection of Plant associated bacteria), France; CNUPBL: Chungbuk National University Plant Bacteriology and Molecular Genetics Lab, Republic of Korea; IPO: Instituut voor plantenziektenkundig onderzoek (Institute for phytopathological research), The Netherlands; IRRI: International Rice Research Institute, Philippines; LMG: Collection of the Laboratorium voor Microbiologie en Microbiële Genetica, Belgium; LPPA: Laboratory of Phytopathology of the Principado de Asturias, Spain; and NCPBP, National Collection of Plant Pathogenic Bacteria, UK. na: not applicable.

^b *C. michiganensis* subsp. *californiensis* subsp. nov. and *C. michiganensis* subsp. *chilensis* subsp. nov. were reported by Yasuhara-Bell and Alvarez (2015) and *C. michiganensis* subsp. *phaseoli* subsp. nov. was reported by González and Trapiello (2014).

MS medium and checked for oxidation/fermentation. Twenty-seven samples of presumed *C. michiganensis* subsp. *nebraskensis* fermented glucose, suggesting that these isolates were misidentified. Sample DP172 exhibited no colony growth and was therefore excluded from all identification tests (Table 2).

All known bacterial control strains and presumptive nonfermenting *C. michiganensis* subsp. *nebraskensis* ($n = 47$) were checked with the ImmunoStrip assay (Tables 1 and 2). All known *C. michiganensis* subspecies, as well as *Microbacterium* sp. and *Ochrobactrum* sp., gave positive reactions with the ImmunoStrip (Table 1). Twenty-six presumptive *C. michiganensis* subsp. *nebraskensis* strains reacted positively with the ImmunoStrip (Table 2). Thirty-five presumptive *C. michiganensis* subsp. *nebraskensis* strains were identified based on 16S rDNA sequences. The remaining 12 presumptive *C. michiganensis* subsp. *nebraskensis* isolates were not tested by sequence analyses, as colony growth was observed much later and they were missed during initial screening. BLAST analysis of these 35 strains revealed that 20 were *Pseudomonas*, two were *Curtobacterium*, one was *Klebsiella*, and one was *Microbacterium* (Table 2). The remaining 11 strains were *Clavibacter* based on 16S rDNA analysis and further characterized by *dnaA* sequence analysis. NJ analysis grouped these 11 strains with the four known *C. michiganensis* subsp. *nebraskensis* (20037, 200800460, NCPPB 2579, and NCPPB 2581) strains (Fig. 2). MP analysis produced a tree with congruent topology (Supplementary Fig. S1).

Loop-mediated isothermal amplification. The LAMP reaction described here was tested on known strains, as well as all strains isolated from diseased maize. The three known *C. michiganensis* subsp. *nebraskensis* strains (20037, 200800460, and NCPPB 2579) (Table 1) and the 23 *C. michiganensis* subsp. *nebraskensis* strains isolated from diseased maize during the 2014 growing season (Table 2) produced positive reactions with the LAMP assay. The 40 leaf samples from the 2015 growing season all produced positive reactions with the LAMP assay (Table 3). The LAMP assay did not react with the negative controls or any non-*C. michiganensis* subsp. *nebraskensis* strains and nonsymptomatic leaf samples (Tables 1, 2, and 3). The described LAMP assay was sensitive to approximately 5 pg of DNA per reaction. LAMP-positive samples also were tested using a handheld real-time fluorescence monitoring device (SMART-DART) and equivalent results were obtained (data not shown).

DISCUSSION

PCR-based techniques used to detect and differentiate closely related species often involve gel electrophoresis and/or DNA sequence analysis, making protocols laborious, time consuming, and costly. Data analysis often requires additional expertise and results are subjective, as determining proper band size on a gel and choosing the right genes to sequence, which analysis to perform,

TABLE 1. (continued from preceding page)

Strain ID	Other ID ^a	Organism ^b	ImmunoStrip	LAMP
A5420	CFBP 4178	<i>D. paradisiaca</i>	—	—
A5579	IPO 2127	<i>D. paradisiaca</i>	—	—
A5688	CFBP 3691	<i>D. paradisiaca</i>	—	—
A6064	CFBP 3696	<i>D. paradisiaca</i>	—	—
A6065	CFBP 1445	<i>D. paradisiaca</i>	—	—
A5581	IPO 2187	<i>D. solani</i>	—	—
A5582	IPO 2188	<i>D. solani</i>	—	—
A5417	CFBP 1278	<i>Dickeya</i> sp.	—	—
A5421	CFBP 1272	<i>Dickeya</i> sp.	—	—
A6066	CFBP 1889	<i>Dickeya</i> sp.	—	—
A6067	CFBP 1890	<i>Dickeya</i> sp.	—	—
A5422	CFBP 2052	<i>D. zeae</i>	—	—
A5423	CFBP 6466	<i>D. zeae</i>	—	—
A5647	CFBP 1531	<i>D. zeae</i>	—	—
A6068	CFBP 1533	<i>D. zeae</i>	—	—
A6069	CFBP 1277	<i>D. zeae</i>	—	—
A6070	CFBP 1884	<i>D. zeae</i>	—	—
A6071	CFBP 1871	<i>D. zeae</i>	—	—
A6072	CFBP 3804	<i>D. zeae</i>	—	—
A6073	CFBP 1447	<i>D. zeae</i>	—	—
A6074	CFBP 3707	<i>D. zeae</i>	—	—
A6075	CFBP 1502	<i>D. zeae</i>	—	—
A3131	ATCC 13048	<i>Enterobacter aerogenes</i>	—	—
A5149	B193	<i>Enterobacter cloacae</i>	—	—
A6091	LJH 1947	<i>Escherichia coli</i>	—	—
A6125	S-3	<i>Klebsiella oxytoca</i>	—	—
A6133	ATCC 13883	<i>K. pneumoniae</i>	—	—
A6126	S-4	<i>K. variicola</i>	—	—
A5726	T3	<i>Microbacterium</i> sp.	+	—
A5724	T1	<i>Ochrobactrum</i> sp.	+	—
A6140	CC27	<i>Pantoea agglomerans</i>	—	—
A1848	IPM 61	<i>Pectobacterium atrosepticum</i>	—	—
A1850	IPM 1260	<i>Pectobacterium atrosepticum</i>	—	—
A1089	QR-11; ATCC 15359	<i>Pectobacterium carotovorum</i>	—	—
A1847	IPM 60	<i>Pectobacterium carotovorum</i>	—	—
A5359	na	<i>Pectobacterium carotovorum</i>	—	—
A5368	5X	<i>Pectobacterium carotovorum</i>	—	—
A5371	CC26	<i>Pectobacterium carotovorum</i>	—	—
A5312	3-12A	<i>Pectobacterium wasabiae</i>	—	—
A3275	A811-1	<i>Pseudomonas fluorescens</i>	—	—
A1151	ATCC 12975	<i>Rhodococcus fasciens</i>	—	—
A3639	E1	<i>Rhodococcus fasciens</i>	—	—
A3643	A1	<i>Rhodococcus fasciens</i>	—	—
A3645	na	<i>Rhodococcus fasciens</i>	—	—
A3292	GMI1000	<i>Ralstonia solanacearum</i>	—	—

TABLE 2. Presumptive *Clavibacter michiganensis* subsp. *nebraskensis* isolates from diseased maize (2014 growing season) used in this study, with associated isolation, identification, ImmunoStrip, and loop-mediated amplification (LAMP) data^a

Strain ID ^b	Location ^c	ID ^d	ImmunoStrip	LAMP
DP101A	Kossuth Co., IA	<i>Microbacterium</i> sp.	+	–
DP101B ^e		nt	+	+
DP103	Kossuth Co., IA	Non- <i>Clavibacter</i> (fermenter)	nt	–
DP104	Beaver Co., OK	<i>C. michiganensis</i> subsp. <i>nebraskensis</i>	+	+
DP105A	Kossuth Co., IA	Non- <i>Clavibacter</i> (fermenter)	nt	–
DP105B		Non- <i>Clavibacter</i> (fermenter)	nt	–
DP105C ^e		nt	+	+
DP107A	Polk Co., NE	Non- <i>Clavibacter</i> (fermenter)	nt	–
DP107B		<i>Pseudomonas</i> sp.	–	–
DP108	Phelps Co., NE	<i>Pseudomonas</i> sp.	–	–
DP109A	Hansford Co., TX	<i>Pseudomonas</i> sp.	–	–
DP109B		<i>Pseudomonas</i> sp.	–	–
DP112A	Webster Co., IA	<i>Pseudomonas</i> sp.	–	–
DP112B		<i>Pseudomonas</i> sp.	–	–
DP113	Clayton Co., IA	<i>Pseudomonas</i> sp.	–	–
DP114A	Hansford Co., TX	<i>C. michiganensis</i> subsp. <i>nebraskensis</i>	+	+
DP114B		Non- <i>Clavibacter</i> (fermenter)	nt	–
DP115A	Marshall Co., IA	<i>C. michiganensis</i> subsp. <i>nebraskensis</i>	+	+
DP115B		Non- <i>Clavibacter</i> (fermenter)	nt	–
DP116	Thayer Co., NE	Non- <i>Clavibacter</i> (fermenter)	nt	–
DP117A	Dodge Co., NE	<i>C. michiganensis</i> subsp. <i>nebraskensis</i>	+	+
DP117B		Non- <i>Clavibacter</i> (fermenter)	nt	–
DP118A	Yuma Co., CO	Non- <i>Clavibacter</i> (fermenter)	nt	–
DP118B		Non- <i>Clavibacter</i> (fermenter)	nt	–
DP119A	Adams Co., NE	<i>Pseudomonas</i> sp.	–	–
DP119B		Non- <i>Clavibacter</i> (fermenter)	nt	–
DP120A	Nuckolls Co., NE	Non- <i>Clavibacter</i> (fermenter)	nt	–
DP120B		Non- <i>Clavibacter</i> (fermenter)	nt	–
DP121A	Castro Co., TX	<i>C. michiganensis</i> subsp. <i>nebraskensis</i>	+	+
DP121B		<i>Pseudomonas</i> sp.	–	–
DP122A	Clay Co., NE	<i>C. michiganensis</i> subsp. <i>nebraskensis</i>	+	+
DP122B		<i>Curtobacterium</i> sp.	+	–
DP132A	Finney Co., KS	Non- <i>Clavibacter</i> (fermenter)	nt	–
DP132B ^e		nt	+	+
DP133A	Emmet Co., IA	Non- <i>Clavibacter</i> (fermenter)	nt	–
DP133B ^e		nt	+	+
DP134	Dallam Co., TX	<i>C. michiganensis</i> subsp. <i>nebraskensis</i>	+	+
DP135A	Stafford Co., KS	Non- <i>Clavibacter</i> (fermenter)	nt	–
DP135B ^e		nt	+	+
DP136	Nodaway Co., MO	<i>Curtobacterium</i> sp.	+	–
DP137	Dallam Co., TX	<i>C. michiganensis</i> subsp. <i>nebraskensis</i>	+	+
DP138A	Dunn Co., WI	Non- <i>Clavibacter</i> (fermenter)	nt	–
DP138B ^e		nt	+	+
DP139A	Dallam Co., TX	<i>Pseudomonas</i> sp.	–	–
DP139B		<i>C. michiganensis</i> subsp. <i>nebraskensis</i>	+	+
DP140A	Howard Co., IA	<i>Klebsiella</i> sp.	–	–
DP140B ^e		nt	+	+
DP142A	Winneshieck Co., IA	Non- <i>Clavibacter</i> (fermenter)	nt	–
DP142B ^e		nt	+	+
DP143A	Union Co., NM	Non- <i>Clavibacter</i> (fermenter)	nt	–
DP143B ^e		nt	+	+
DP144A	Linn Co., IA	Non- <i>Clavibacter</i> (fermenter)	nt	–
DP144B		Non- <i>Clavibacter</i> (fermenter)	nt	–
DP144C ^e		nt	+	+
DP148A	Mitchell Co., IA	Non- <i>Clavibacter</i> (fermenter)	nt	–
DP148B		Non- <i>Clavibacter</i> (fermenter)	nt	–
DP149A	Moore Co., TX	Non- <i>Clavibacter</i> (fermenter)	nt	–
DP149B ^e		nt	+	+
DP150A	St. Charles Co., MO	Non- <i>Clavibacter</i> (fermenter)	nt	–
DP150B		Non- <i>Clavibacter</i> (fermenter)	nt	–
DP150C ^e		nt	+	+
DP157	Moore Co., TX	Non- <i>Clavibacter</i> (fermenter)	nt	–
DP160	Yuma Co., CO	<i>Pseudomonas</i> sp.	–	–
DP162A	Codington Co., SD	<i>Pseudomonas</i> sp.	–	–
DP162B		<i>Pseudomonas</i> sp.	–	–
DP162C		<i>Pseudomonas</i> sp.	–	–
DP163	Codington Co., SD	<i>Pseudomonas</i> sp.	–	–
DP164	Cedar Co., NE	<i>C. michiganensis</i> subsp. <i>nebraskensis</i>	+	+
DP165A	Fayette Co., IA	<i>Pseudomonas</i> sp.	–	–
DP165B		<i>Pseudomonas</i> sp.	–	–
DP165C		<i>C. michiganensis</i> subsp. <i>nebraskensis</i>	+	+
DP172	White Co., IN	No colony growth	nt	nt
DP173	Norton Co., KS	<i>Pseudomonas</i> sp.	–	–
DP174A	Yuma Co., CO	<i>Pseudomonas</i> sp.	–	–
DP174B		<i>Pseudomonas</i> sp.	–	–

^a nt = not tested.

^b Letter denotes different colony type obtained from same sample ID.

^c Location of sample indicated by county and state.

^d Identification based on bacterial identification tests described in the Materials and Methods.

^e *C. michiganensis* subsp. *nebraskensis* colonies observed after initial screening and no sequence analyses were performed.

TABLE 3. Putative *Clavibacter michiganensis* subsp. *michiganensis*-infected corn leaf tissue samples used in this study (growing season 2015), with associated identification, enzyme-linked immunosorbent assay (ELISA), and loop-mediated amplification (LAMP) data

Number	Sample ID	Presumptive ID ^a	Location ^b	Collection date	ELISA test ^c	LAMP
1	3007F04 ^d	<i>C. michiganensis</i> subsp. <i>nebraskensis</i>	Haskell Co., KS	06/12/2015	+	+
2	3007F05	<i>Xanthomonas campestris</i>	Furnas Co., NE	06/30/2015	N/A	–
3	3007F06	<i>X. campestris</i>	Fillmore Co., NE	06/22/2015	N/A	–
4	3007F07 ^d	<i>C. michiganensis</i> subsp. <i>nebraskensis</i>	Fillmore Co., NE	06/22/2015	+	+
5	3007F08	<i>X. campestris</i>	York Co., NE	06/22/2015	N/A	–
6	3007F09 ^d	<i>C. michiganensis</i> subsp. <i>nebraskensis</i>	Gray Co., KS	06/22/2015	+	+
7	3007F10	<i>X. campestris</i>	Clay Co., NE	06/27/2015	N/A	–
8	3007G01	<i>X. campestris</i>	Clay Co., NE	06/29/2015	N/A	–
9	3007G02	<i>X. campestris</i>	Hamilton Co., NE	06/29/2015	N/A	–
10	3007G03	<i>X. campestris</i>	York Co., NE	06/29/2015	N/A	–
11	3007G04 ^d	<i>C. michiganensis</i> subsp. <i>nebraskensis</i>	Platte Co., NE	07/01/2015	+	+
12	3007G05 ^d	<i>C. michiganensis</i> subsp. <i>nebraskensis</i>	Platte Co., NE	06/30/2015	+	+
13	3007G06	<i>X. campestris</i>	Fillmore Co., NE	06/30/2015	N/A	–
14	3007G07 ^d	<i>C. michiganensis</i> subsp. <i>nebraskensis</i>	Howard Co., NE	07/01/2015	+	+
15	3007G08 ^d	<i>C. michiganensis</i> subsp. <i>nebraskensis</i>	Fillmore Co., NE	06/30/2015	+	+
16	3007G09 ^d	<i>C. michiganensis</i> subsp. <i>nebraskensis</i>	Saline Co., NE	06/29/2015	+	+
17	3007G10	<i>X. campestris</i>	Chase Co., NE	07/01/2015	N/A	–
18	3008A01	<i>X. campestris</i>	Phillips Co., CO	06/30/2015	N/A	–
19	3008A02	<i>X. campestris</i>	Antelope Co., NE	07/06/2015	N/A	–
20	3008A03	<i>X. campestris</i>	Sherman Co., NE	07/03/2015	N/A	–
21	3008A04 ^d	<i>C. michiganensis</i> subsp. <i>nebraskensis</i>	Hartley Co., TX	07/07/2015	+	+
22	3008A09 ^d	<i>C. michiganensis</i> subsp. <i>nebraskensis</i>	York Co., NE	07/08/2015	+	+
23	3008C01 ^d	<i>C. michiganensis</i> subsp. <i>nebraskensis</i>	Marion Co., IA	07/13/2015	+	+
24	3008C02 ^d	<i>C. michiganensis</i> subsp. <i>nebraskensis</i>	Dakota Co., MN	07/13/2015	+	+
25	3008C03	<i>Cercospora zeae-maydis</i>	Jennings Co., IN	07/13/2015	N/A	–
26	3005F04	<i>Exserohilum turcicum</i>	Otsego Co., NY	10/21/2013	N/A	–
27	3005F05	<i>Exserohilum turcicum</i>	Madison Co., NY	10/21/2013	N/A	–
28	3008C07 ^d	<i>C. michiganensis</i> subsp. <i>nebraskensis</i>	Nobles Co., MN	07/15/2015	+	+
29	3008C08	<i>X. campestris</i>	Lincoln Co., NE	07/14/2015	N/A	–
30	3008C09	<i>X. campestris</i>	York Co., NE	07/14/2015	N/A	–
31	3008C10	<i>X. campestris</i>	Yuma Co., CO	07/11/2015	N/A	–
32	3008F05	<i>X. campestris</i>	Clay Co., NE	07/20/2015	NA	–
33	3008F06 ^d	<i>C. michiganensis</i> subsp. <i>nebraskensis</i>	Cuming Co., NE	07/16/2015	+	+
34	3008F07	<i>X. campestris</i>	Nuckolls Co., NE	07/20/2015	N/A	–
35	3008F08	<i>X. campestris</i>	Perkins Co., NE	07/22/2015	N/A	–
36	3008F10	<i>X. campestris</i>	Hayes Co., NE	07/20/2015	N/A	–
37	3008G01	<i>X. campestris</i>	Chase Co., NE	07/20/2015	N/A	–
38	3008G02	<i>X. campestris</i>	Keith Co., NE	07/22/2015	N/A	–
39	3008G03	<i>X. campestris</i>	Keith Co., NE	07/22/2015	N/A	–
40	3008G04	<i>X. campestris</i>	York Co., NE	07/21/2015	N/A	–
41	3008G05 ^d	<i>C. michiganensis</i> subsp. <i>nebraskensis</i>	Dakota Co., MN	07/20/2015	+	+
42	3008G06 ^d	<i>C. michiganensis</i> subsp. <i>nebraskensis</i>	Dakota Co., MN	07/20/2015	+	+
43	3008G10 ^d	<i>C. michiganensis</i> subsp. <i>nebraskensis</i>	Watonswan Co., MN	07/25/2015	+	+
44	3009C05 ^d	<i>C. michiganensis</i> subsp. <i>nebraskensis</i>	Dickinson Co., IA	07/28/2015	+	+
45	3009C06 ^d	<i>C. michiganensis</i> subsp. <i>nebraskensis</i>	Sherman Co., KS	07/23/2015	+	+
46	3009C09 ^d	<i>C. michiganensis</i> subsp. <i>nebraskensis</i>	Hartley Co., TX	08/03/2015	+	+
47	3009F05 ^d	<i>C. michiganensis</i> subsp. <i>nebraskensis</i>	Dakota Co., MN	08/08/2015	+	+
48	3009F06 ^e	<i>C. michiganensis</i> subsp. <i>nebraskensis</i>	Dickinson Co., IA	08/03/2015	+	+
49	3009F07 ^e	<i>C. michiganensis</i> subsp. <i>nebraskensis</i>	Winneshiek Co., IA	08/05/2015	+	+
50	3009F08 ^e	<i>C. michiganensis</i> subsp. <i>nebraskensis</i>	Dekalb Co., IL	08/03/2015	+	+
51	3009F09 ^e	<i>C. michiganensis</i> subsp. <i>nebraskensis</i>	Dekalb Co., IL	08/03/2015	+	+
52	3009G01 ^e	<i>C. michiganensis</i> subsp. <i>nebraskensis</i>	LaSalle Co., IL	08/05/2015	+	+
53	3009G04 ^e	<i>C. michiganensis</i> subsp. <i>nebraskensis</i>	Tripp Co., SD	08/10/2015	+	+
54	3009G05 ^e	<i>C. michiganensis</i> subsp. <i>nebraskensis</i>	Cedar Co., NE	08/10/2015	+	+
55	3009G07 ^e	<i>C. michiganensis</i> subsp. <i>nebraskensis</i>	Boone Co., NE	08/12/2015	+	+
56	3009G09 ^e	<i>C. michiganensis</i> subsp. <i>nebraskensis</i>	Reading Co., MN	08/13/2015	+	+
57	3009G10	<i>Exserohilum turcicum</i>	La Crosse Co., WI	08/12/2015	N/A	–
58	1031G05 ^e	<i>C. michiganensis</i> subsp. <i>nebraskensis</i>	Carroll Co., IL	08/13/2015	+	+
59	1031G07 ^e	<i>C. michiganensis</i> subsp. <i>nebraskensis</i>	Dakota Co., NE	08/13/2015	+	+
60	1031G08 ^e	<i>C. michiganensis</i> subsp. <i>nebraskensis</i>	Dakota Co., NE	08/13/2015	+	+
61	1031G09 ^e	<i>C. michiganensis</i> subsp. <i>nebraskensis</i>	Hartley Co., TX	08/16/2015	+	+
62	1032A04 ^e	<i>C. michiganensis</i> subsp. <i>nebraskensis</i>	Gove Co., KS	08/18/2015	+	+
63	1032A05 ^e	<i>C. michiganensis</i> subsp. <i>nebraskensis</i>	Osceola Co., IA	08/17/2015	+	+
64	1032A06 ^e	<i>C. michiganensis</i> subsp. <i>nebraskensis</i>	Nemaha Co., KS	08/19/2015	+	+
65	1032A07 ^e	<i>C. michiganensis</i> subsp. <i>nebraskensis</i>	Dubuque Co., IA	08/17/2015	+	+
66	Unnamed ^d	<i>C. michiganensis</i> subsp. <i>nebraskensis</i>	Doniphan Co., KS	08/24/2015	+	+
67	1032A09 ^e	<i>C. michiganensis</i> subsp. <i>nebraskensis</i>	Hughes Co., SD	08/19/2015	+	+

^a Presumptive identification made by DuPont Pioneer based on disease symptoms and commercially available serological tests (bacteria diagnoses).

^b Location of sample indicated by county and state.

^c ELISA tests include Agdia ImmunoStrip (Cmm) test and Neogen ELISA (Cmm) test. N/A = not applicable.

^d Agdia ImmunoStrip (Cmm).

^e Neogen ELISA (Cmm) test.

and how phylogenetic trees are displayed are user-dependent. These methods are impractical for routine diagnosis due to sheer volume of samples and expense of time and money. Real-time PCR can eliminate the need for gel analyses but requires expensive thermocycling equipment with real-time fluorescence monitoring capabilities.

The LAMP assay is an isothermal nucleic acid amplification technology that has numerous advantages, including eliminating the need for expensive thermocycling equipment, as required for PCR. LAMP reactions can be confirmed visually at end-point via turbidity, making it possible to run reactions with only a water bath that can maintain a constant temperature of 65°C (Labarre et al. 2010; Mori et al. 2001), thereby dramatically reducing costs. The isothermal nature of LAMP makes it ideal as a field diagnostic. Detection of LAMP products is also possible using several other formats, such as gel electrophoresis, real-time turbidimetry, and end-point or real-time fluorescence monitoring (Kubota et al. 2011; Mori et al. 2006; Mori et al. 2004; Mori et al. 2001; Tomita et al. 2008). Hand-held real-time fluorescence monitoring devices are available, such as the SMART-DART (Jenkins et al. 2011), which can allow for field diagnoses in real-time.

LAMP technology is publically available, making it the most widely researched isothermal amplification technology (Craw and Balachandran 2012). As LAMP has been well-characterized, troubleshooting support during the development process is easily accessible. LAMP performs equal to or better than equivalent PCR-, immunoassay-, or culture-based detection methods, and sensitivity has been shown to exceed that of equivalent PCR assays (Fujino et al. 2005). The nature of LAMP primers' recognizing six unique sequences affords it a greater sensitivity. Additionally, LAMP reactions are faster than conventional PCR because time is not lost cycling between different denaturing, annealing, and elongation temperatures, with majority of reactions starting exponentially at 20 to 30 min and requiring a maximum reaction time ≤1 h.

A LAMP-based assay developed for specific detection of the Goss's wilt pathogen, *C. michiganensis* subsp. *nebraskensis*, was successful in identifying the target pathogen and distinguishing it from other *C. michiganensis* subspecies and nontarget genera. Whole-genome sequence comparisons identified potential gene targets unique enough to enable specific detection of *C. michiganensis* subsp. *nebraskensis* and subspecies differentiation. LAMP primers were designed to detect the TRAP-type C4-dicarboxylate transport system large permease component. BLAST analysis of this gene resulted in matches only to the published *C. michiganensis* subsp. *nebraskensis* NCPPB 2581 genome sequence (HE614873.1), suggesting high specificity. This approach for selecting the appropriate molecular target for LAMP primer development has been successful with other bacteria (Yasuhara-Bell et al. 2013, 2014, 2015a).

The LAMP assay used in this study gave positive reactions for known reference strains of *C. michiganensis* subsp. *nebraskensis*, bacteria isolated from diseased maize that were confirmed as *C. michiganensis* subsp. *nebraskensis* by sequence analyses, and DNA extracts from symptomatic leaf tissue infected with *C. michiganensis* subsp. *nebraskensis*. The ImmunoStrip assay, which was included in this study as a presumptive identification test, was positive with all *C. michiganensis* subsp. *nebraskensis* strains tested, as well as other

C. michiganensis subspecies and a few non-*Clavibacter* strains. All LAMP-positive samples were also positive by immunoassay (ImmunoStrip [all bacterial isolates and 21 leaf samples] and Neogen Cmn ELISA [19 leaf samples]). The LAMP assay had equal performance when used with the hand-held SMART-DART, showing potential for field application.

Forty-two samples from the 2014 growing season were suspected as being *C. michiganensis* subsp. *nebraskensis*, but only 23 were confirmed as *C. michiganensis* subsp. *nebraskensis*, using both 16S rDNA and *dnaA* sequence analyses and the LAMP assay itself. Among the 23 positive cultures, eight isolates were from Iowa (DP101B, DP105C, DP115A, DP133B, DP140B, DP142, DP144C, and DP165C), six were from Texas (DP114A, DP121A, DP134, DP137, DP139B, and DP149B), three were from Nebraska (DP117A, DP122A, and DP164), two were from Kansas (DP132B and DP135B), one sample was from Oklahoma (DP104), one from New Mexico (DP143), one from Wisconsin (DP138B), and one from Missouri (DP50C). It should be noted that inefficient isolation of bacteria from symptomatic leaves, as well as inaccurate selection of bacterial colony types for subsequent testing, may have contributed to the low confirmation rate of the initial diagnosis of these 42 samples. This observation prompted the addition of 40 symptomatic leaf samples from the 2015 growing season for validation testing of

Fig. 1. *Clavibacter michiganensis* subsp. *nebraskensis* NCPPB 2581 tripartite ATP-independent periplasmic (TRAP)-type C4-dicarboxylate transport system large permease component DNA sequence (HE614873.1) with corresponding locations of loop-mediated amplification (LAMP) primers. F1 and B1 denote sequences complementary to the FIP and BIP overhangs, respectively, which allows the formation of the “dumbbell” structure that is the key to the LAMP reaction.

TABLE 4. Loop-mediated amplification primers used in this study^a

Oligonucleotide Primer	Sequence (5'-3')	Source/reference
TRAP-F3	TCCTCTGGCGGCTGGT	This study
TRAP-B3	GGACTCCATCACGTCCTCG	This study
TRAP-FIP	GAACGGCAGGATGTCCGGGTTCTCCTTCGTGCGGAAGC	This study
TRAP-BIP	TGCTGGTGTTCCTCGGGATCGACGCCCGTGAGCGACT	This study
TRAP-Loop	ATCCCGGTGCTCGTCAC	This study
TRAP-Probe	/56-FAM/ACGCTGAGGACCCGGATGCGAATGCGGATGCGGATGCCGAATCCCGGTGCTCGTCAC	This study
Quencher Probe	TCGGCATCCGCATCCGCATTCCGCATCCGGTCTCAGCGT/3BHQ_1/	Kubota et al. 2011

^a /56-FAM/, 5' 6-carboxyfluorescein; /3BHQ_1/, 3' Black Hole Quencher 1.

the LAMP assay, which demonstrated 100% accuracy and confirmation of the initial diagnosis.

The LAMP assay described in this study has potential for direct testing of infected plant tissues in the field (Yasuhara-Bell et al. 2015b). Some initial testing involved a few samples from the

2015 growing season, with leaf tissues cut into small pieces and allowed to incubate at room temperature for a period of 1 to 2 h in 1× Tris-EDTA (TE) buffer. The supernatant was then incubated at 95°C for 10 min and used in the LAMP assay. Several samples yielded positive LAMP results with this method (data not shown),

Fig. 2. Phylogenetic analysis of *dnaA* sequences. Neighbor-joining trees were constructed using the Jukes-Cantor method to compute evolutionary distances and based on partial *dnaA* sequences of 49 *Clavibacter* strains and rooted using *Aquifex aeolicus* (accession AE000657.1) as an outgroup. Sequences were aligned and trimmed to 661 nt. Bootstrap values were generated from 1,000 replications and bootstrap values of 50% or more are shown to the left of corresponding nodes. Cm-Pepper, *C. michiganensis* subsp. from pepper; CmCa, *C. michiganensis* subsp. *californiensis* subsp. nov.; CmCh, *C. michiganensis* subsp. *chilensis* subsp. nov.; Cmi, *C. michiganensis* subsp. *insidiosus*; Cmm, *C. michiganensis* subsp. *michiganensis*; Cmn, *C. michiganensis* subsp. *nebraskensis*; Cmp, *C. michiganensis* subsp. *phaseoli* subsp. nov.; Cms, *C. michiganensis* subsp. *sepedonicus*; and Cmt, *C. michiganensis* subsp. *tessellarius*.

but several others did not. The samples that were tested by this method were dried out; thus, the ability for bacteria to diffuse into the buffer may have been compromised. The ability of LAMP to detect bacteria in plant samples by direct boiling of bacterial suspensions collected by soaking infected tissues has been demonstrated previously (Yasuhara-Bell et al. 2015b). Therefore, further testing of this assay directly from fresh symptomatic leaf tissue without the need for DNA extraction is required before this assay would be ready for direct testing of plant tissues in the field.

Future work should also include assessing potential inhibition from maize tissue samples. Adjusting plant sampling protocols to minimize the release of inhibitors into solution, as well as assessing a potential need for an enrichment step, will allow this test to function as described. Furthermore, incorporation of an internal control in a multiplex reaction will allow this assay to act as a stand-alone test and minimize the need for inclusion of extra controls when running sample tests. Regardless, this LAMP assay can be implemented immediately as a confirmatory test for *C. michiganensis* subsp. *nebraskensis*, following isolation of bacteria on semiselective media, as per standard protocols, or direct testing of DNA extracted from symptomatic leaf tissues. Additionally, the gene used for this LAMP assay can be targeted using all molecular-based detection platforms.

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