PCR-Mediated Detection and Quantification of the Goss's Wilt Pathogen *Clavibacter michiganensis* subsp. *nebraskensis* Via a Novel Gene Target

R. Ryan McNally, Carol A. Ishimaru, and Dean K. Malvick

First, second, and third authors: Department of Plant Pathology, University of Minnesota, St. Paul 55108; and second and third authors: Stakman-Borlaug Center for Sustainable Plant Health, University of Minnesota.
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ABSTRACT

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Goss's leaf blight and wilt of maize (corn) is a significant and reemerging disease caused by the bacterium *Clavibacter michiganensis* subsp. *nebraskensis*. Despite its importance, molecular tools for diagnosing and studying this disease remain limited. We report the identification of CMN_01184 as a novel gene target and its use in conventional PCR (cPCR) and SYBR green-based quantitative PCR (qPCR) assays for specific detection and quantification of *C. michiganensis* subsp. *nebraskensis*. The cPCR and qPCR assays based on primers targeting CMN_01184 specifically amplified only *C. michiganensis* subsp. *nebraskensis* among a diverse collection of 129 bacterial and fungal isolates, including multiple maize

bacterial and fungal pathogens, environmental organisms from agricultural fields, and all known subspecies of *C. michiganensis*. Specificity of the assays for detection of only *C. michiganensis* subsp. *nebraskensis* was also validated with field samples of *C. michiganensis* subsp. *nebraskensis*-infected and uninfected maize leaves and *C. michiganensis* subsp. *nebraskensis*-infested and uninfested soil. Detection limits were determined at 30 and 3 ng of pure *C. michiganensis* subsp. *nebraskensis* DNA, and 100 and 10 CFU of *C. michiganensis* subsp. *nebraskensis* for the cPCR and qPCR assays, respectively. Infection of maize leaves by *C. michiganensis* subsp. *nebraskensis* was quantified from infected field samples and was standardized using an internal maize DNA control. These novel, specific, and sensitive PCR assays based on CMN_01184 are effective for diagnosis of Goss's wilt and for studies of the epidemiology and host-pathogen interactions of *C. michiganensis* subsp. *nebraskensis*.

Clavibacter michiganensis subsp. *nebraskensis* is the causative agent of Goss's leaf blight and wilt (commonly known as Goss's wilt), a destructive and spreading disease of maize (*Zea mays* subsp. *mays*). Symptoms include leaf blight and systemic vascular wilt. The leaf blight develops as long lesions that extend parallel to veins and exhibit irregular, dark, water-soaked spots or 'freckles' (Jackson et al. 2007). Yield losses due to Goss's leaf blight and wilt exceeding 50% have been reported for susceptible hybrids (Jackson et al. 2007; Jardine and Claflin 2016).

Goss's wilt of maize was first identified in the United States in 1969 in Nebraska (Vidaver and Mandel 1974; Wysong et al. 1973). The disease persisted as a sporadic regional issue in Nebraska and bordering states in the following decades (Jackson et al. 2007; Vidaver et al. 1981). More recently, since about 2006, the incidence and severity of Goss's wilt has increased in Nebraska, and the geographic distribution has expanded greatly in the United States and Canada. While factors responsible for this increase remain speculative, to date 16 states and two Canadian provinces have reported Goss's wilt (Friskop et al. 2014; Howard et al. 2015; Korus 2011; Malvick et al. 2010; Ruhl et al. 2009; Singh et al. 2015; Tambong et al. 2015; Wysong et al. 1981). Consequently, Goss's wilt of maize is considered a reemerging and spreading disease of considerable economic importance (Jackson et al. 2007).

The recent increase in Goss's wilt as an economically important disease in North America highlights a need for improved and widely accessible methods for rapid and accurate disease diagnosis and pathogen-specific detection. Diagnosis of Goss's wilt has historically been based on the presence of typical disease symptoms

on leaves and signs of bacterial infection, such as bacterial ooze. Symptoms caused by several other common bacterial and fungal maize diseases can be confused with Goss's wilt. Reported techniques for detection of *C. michiganensis* subsp. *nebraskensis* include isolation on semiselective media, DNA and protein fingerprinting, a quantitative polymerase chain reaction (qPCR) assay that requires a TaqMan probe, and nonspecific serological assays (Bach et al. 2003; Gross and Vidaver 1979; Korus 2011; Lee et al. 1997; Louws et al. 1998; Pastrok and Rainey 1999; Smidt and Vidaver 1986; Zaluga et al. 2011). Recently, a loop-mediated isothermal PCR (LAMP) assay was developed for specific detection of *C. michiganensis* subsp. *nebraskensis* in maize (Yasuhara-Bell et al. 2016). This LAMP assay provides a rapid means of pathogen detection; however, it is not quantitative and LAMP assays are currently used less commonly in diagnostic and research laboratories than other PCR-based assays.

PCR techniques have proven to be powerful and readily accessible methods for detection, identification, quantification, and diagnosis of bacterial and other plant pathogens and diseases (Schaad and Frederick 2002; Vincelli and Tisserat 2008). Conventional PCR (cPCR) is simple, fast, specific, doesn't require expensive equipment, and eliminates the need for selective media or culturing of an organism to confirm its presence. Real-time qPCR generally has high sensitivity, allows for enumeration, requires no gel analysis, has high throughput potential, and can specifically detect multiple different organisms simultaneously in one test (Bilodeau 2011; McCartney et al. 2003; Mumford et al. 2006; Schaad and Frederick 2002; Vincelli and Tisserat 2008). qPCR can accurately determine the copy number of a specific target gene or DNA molecule from a bacterial or fungal species, which can be correlated to microbial number or population (Gachon and Saindrenan 2004; Schaad and Frederick 2002; Schroeder et al. 2006; Vincelli and Tisserat 2008).

To date, methods for specific detection and quantification of *C. michiganensis* subsp. *nebraskensis* have not been reported that

Corresponding author: D. K. Malvick; E-mail address: dmalvick@umn.edu

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allow use of cPCR or qPCR not requiring expensive TaqMan probes for qPCR, two criteria that make assays readily accessible and adoptable for many laboratories. A challenge in developing specific assays has been the fact that *C. michiganensis* subsp. *nebraskensis* is one among eight closely related subspecies of *C. michiganensis* pathogens affecting a wide diversity of crops, (Eichenlaub and Gartemann 2011). The genetic relatedness within the species has made accurate and specific detection of *C. michiganensis* subsp. *nebraskensis* difficult (González and Trapiello 2014; Korus 2011; Yasuhara-Bell et al. 2016). We recognized the potential of cPCR and qPCR for specific, sensitive, and high throughput detection and quantification of *C. michiganensis* subsp. *nebraskensis* in maize tissues and soil and for improving diagnosis of Goss's wilt. The objective of this work was to capitalize on this potential and to develop and validate assays that meet these technical and biological criteria.

Here, we report widely adaptable and cost-effective techniques for sensitive and specific detection and quantification of the Goss's wilt pathogen *C. michiganensis* subsp. *nebraskensis*. Using a novel

gene target specific to *C. michiganensis* subsp. *nebraskensis*, both qualitative (cPCR) and SYBR green-based (qPCR) assays were developed for the identification, detection, and enumeration of *C. michiganensis* subsp. *nebraskensis* populations in vitro and in maize leaves and complex soil environments. The new assays represent novel and important tools for diagnosis and rapid detection of *C. michiganensis* subsp. *nebraskensis* and for applications in epidemiological, plant-microbe interaction, and host resistance research.

MATERIALS AND METHODS

Source and identity of isolates. The 129 bacterial and fungal isolates used in this study are listed in Table 1. The collection represented 28 species and 20 genera comprising diverse groups of bacteria and fungi. In total, 78 isolates of *C. michiganensis* were used, including 35 isolates of *C. michiganensis* subsp. *nebraskensis* collected at different times and from different geographic locations in the midwestern United States. *C. michiganensis* subsp. *nebraskensis*

TABLE 1. Bacterial and fungal isolates used to evaluate specificity of conventional and quantitative polymerase chain reaction (PCR) assays for the detection of *C. michiganensis* subsp. *nebraskensis* based on gene CMN_01184

Species	Host of origin	No. Isolates	CMN_01184 detection		Source ^c
			Conventional PCR ^a	qPCR ^b	
<i>C. michiganensis</i> subsp. <i>nebraskensis</i>	Maize	35	+	+	1,2,3,4,5,6,7
<i>C. michiganensis</i> subsp. <i>michiganensis</i>	Tomato	19	–	–	2,8
<i>C. michiganensis</i> subsp. <i>sepedonicus</i>	Potato	16	–	–	2
<i>C. michiganensis</i> subsp. <i>insidiosus</i>	Alfalfa	2	–	–	2
<i>C. michiganensis</i> subsp. <i>tessellarius</i>	Wheat	2	–	–	2
<i>C. michiganensis</i> subsp. <i>phaseoli</i>	Bean	1	–	–	9
<i>C. michiganensis</i> subsp. <i>californiensis</i>	Tomato	2	–	–	4
<i>C. michiganensis</i> subsp. <i>chilensis</i>	Pepper	1	–	–	4
<i>Acidovorax avenae</i>	Crab grass	1	–	–	2
<i>Agrobacterium tumefaciens</i>	Tomato	2	–	–	2
<i>Bacillus cereus</i>	Alfalfa	1	–	–	2
<i>Bacillus subtilis</i>	Environment	2	–	–	2
<i>Burkholderia andropogonis</i>	Maize	1	–	–	10
<i>Brevibacterium lactifermentum</i>	Environment	1	–	–	2
<i>Corynebacterium callunae</i>	Environment	1	–	–	2
<i>Corynebacterium glutamicum</i>	Environment	1	–	–	2
<i>Curtobacterium flaccumfacians</i>	Beet	3	–	–	2
<i>Dickeya zeae</i>	Maize	2	–	–	2
<i>Enterobacter cloacae</i>	Environment	1	–	–	2
<i>Exserohilum turcicum</i>	Corn	2	–	–	11
<i>Fusarium graminearum</i>	Maize	2	–	–	12
<i>Fusarium oxysporum</i>	Soybean	2	–	–	7
<i>Fusarium solani</i>	Potato, soybean	3	–	–	7
<i>Fusarium virguliforme</i>	Soybean	1	–	–	7
<i>Micrococcus luteus</i>	Environment	1	–	–	2
<i>Pantoea agglomerans</i>	Apple	3	–	–	2
<i>Pantoea stewartii</i>	Maize	3	–	–	13,14
<i>Pectobacterium carotovora</i>	Potato	4	–	–	2
<i>Pseudomonas fluorescens</i>	Environment	2	–	–	2
<i>Pseudomonas syringae</i>	Bean, tomato	5	–	–	2
<i>Pythium dissotium</i>	Soybean	1	–	–	7
<i>Rhizoctonia solani</i>	Soybean	1	–	–	7
<i>Streptomyces griseus</i>	Environment	1	–	–	2
<i>Xanthomonas campestris</i>	Sorghum	2	–	–	2
<i>Xanthomonas</i> spp.	Foxtail	2	–	–	2
Total		129			

^a + = expected 0.67-kb PCR product band; – = no PCR product detected.

^b + = samples with cycle threshold (Ct) values ranging from 14.2 to 24.5; – = samples with Ct values greater than 35, which were considered to have nondetected DNA.

^c Isolates and DNA were provided by the following sources. 1) A. Vidaver: isolates 20032, 2006ScottsBluff-3, 200900798, CN18-5, CN15-1, CN18-1, CN28-1, CN30-1, CN72-28, CN76-2, 15-2.0, 15-2.1, 15-2.2, 156-2.0, 156-2.2, 82055, 82055.9, CIGP010, PCC1, CB101.0, CP1.0; 2) C. Ishimaru: isolates CIC056, CIC057, CIC058, CIC059, CIC251, CIC252, FGI-12, LK1-12, MS1-12, MS2-12, CIC190, S26, 10 D, 11J, 6E, ATCC33113, CIC001, CIC003, CIC005, CIC031, CIC077, CIC144, CIC226, CIC227, CIC228, CIC229, S5, TS4, CIC266, CICR1-3, CIA003, CIA004, UW85, CIGP012, ATCC9372, CIC188, CIR294, CIR295, DF13, CIGP008, C9-1, 36740, Lee R-49a, CIP048, B45, CIP054, CIGP013, CIX012, CIX014, CIX001, CIX007, BC1-11A, ED1-11A, PK1-11A, 3404 VS-1, CIP 051, CIG 014, B-37b, C-9-96b, C9-1, Eh75115, A506, 31a, L113-7; 3) C. Bradley: isolates CIC344, CIC345; 4) A. Alvarez: isolates K087, C55, C66, ZUM3936; 5) UMN Plant Disease Clinic: isolates PDC4-11A, PDC4-12, PDC5-11A, PDC5-12, PDC7-12, CIC334; 6) Kasia Kinzer: isolates PP1201420, PP1201528, PP1201626, PP1301135; 7) D. Malvick: isolates 07-071, 07-321, 07-154, 07-373, 07-500, Fv1, MN24JJ, Rh; 8) G. Coaker: isolates AZ28, CA0002, CA498, CASJ009, CFBP7491, CFBP8017, CFBP8019, CMM5, HZII12, NZ2541, Stock20; 9) A. Gonzalez: isolate LPPA982; 10) S. Bissonnette; 11) O. Fajolu: Et race 0, Et race 123N; 12) R. Dill-Macky: isolates 1-1, 14-1-2; 13) S. Miller: isolates Pstew1, Pstew2; 14) G. Beattie: isolates: ES Rif9A.

isolates were identified via colony morphology and were confirmed using either 16S rDNA sequencing, a commercially available *Clavibacter michiganensis*-specific ImmunoStrip (Agdia Inc., Elkhart, IN) assay, or both, in conjunction with greenhouse pathogenicity studies. *C. michiganensis* subsp. *nebraskensis* morphology was characterized by apricot-orange pigmentation, circular colony type, and slow growth (about 5 days). Control isolates for specificity testing were plant pathogens and environmental isolates from agricultural fields, including fungal and bacterial maize pathogens and nonmaize pathogens, fungi, and gram-negative and gram-positive bacteria.

DNA extraction from bacteria and fungi. *C. michiganensis* subsp. *nebraskensis* isolates were streaked on nutrient broth yeast extract (NBY) agar amended with MgSO₄ (2 mM) and cycloheximide (350 µM) and were grown at 25°C for 5 days (Vidaver 1967). *C. michiganensis* subsp. *nebraskensis* cells were harvested using a sterile cotton swab and were incubated in lysozyme buffer (20 mg/ml) prior to DNA extraction, using a DNeasy blood and tissue kit adapted for gram-positive bacteria as described by the manufacturer (Qiagen, Valencia, CA). Fungal DNA was extracted from mycelia grown on nutrient broth medium. Mycelia were harvested using a sterilized cotton swab and scoopula and were homogenized in a FastPrep instrument (QBiogene, Irvine, CA) and DNA was extracted, using a FastPrep FastDNA kit (MP Biomedicals, Solon, OH), according to the manufacturer's instructions for fungi. Quality and quantity of DNA was determined for all samples using a Nanodrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA). DNA was stored at -20°C.

Comparative genomics and primer design. Potential gene targets for specific detection of *C. michiganensis* subsp. *nebraskensis* were identified by conducting a comparative genomic analysis. The annotated genome of *C. michiganensis* subsp. *nebraskensis* NCPPB 2581 (National Center for Biotechnology Information [NCBI]: NC_020891) was manually filtered relative to predicted orthology in the genomes of *C. michiganensis* subsp. *michiganensis* NCPPB 382 (NCBI: NC_009480) and *C. michiganensis* subsp. *sepedonicus* ATCC 33113 (NCBI: NC_010407). Putative singletons were discovered via the NCBI sequence alignment search tools BLASTN, BLASTX, and CD-search. Then, putative gene targets for *C. michiganensis* subsp. *nebraskensis* detection were filtered relative to nucleic acid sequence conservation with other *C. michiganensis* subspecies. Genes meeting the following criteria were considered putative targets for *C. michiganensis* subsp. *nebraskensis* detection: i) no nucleic acid or amino acid sequence conservation with other *C. michiganensis* subspecies and ii) limited nucleic acid sequence conservation with other bacteria and fungi. Based on these analyses, CMN_01184 was identified as a target for specific detection of *C. michiganensis* subsp. *nebraskensis*. Predicted orthologs in *C. michiganensis* were also analyzed for homology in other bacterial species, to identify a target for general detection of all *C. michiganensis* subspecies. The predicted transporter CMN_00971 (NCBI: CMN_RS04740) was identified in *C. michiganensis* subsp. *nebraskensis* NCPPB 2581 and was subjected to BLASTN analysis, with complete query coverage ranging from 96 to 98% nucleic acid

sequence conservation, to evaluate the potential of CMN_00971 (390 bp) for detection of all *C. michiganensis* subspecies.

Forward and reverse primers were created for specific *C. michiganensis* subsp. *nebraskensis* detection based on the nucleic acid sequences of CMN_01184 (NCBI: CMN_RS05785) and CMN_00971 (NCBI: CMN_RS04740). Primers for cPCR were designed manually. Primers for qPCR were created using default parameters with Primer Express software (Applied Biosystems, Foster City, CA). Primers for general bacterial detection and maize DNA quantification were used as previously described (Hernández et al. 2004; Monciardini et al. 2002). All primers used in this study are listed and described in Table 2.

Specificity of cPCR and qPCR assays. Primer set 1184-F/R was tested using cPCR on DNA from 129 isolates of plant- and soil-associated bacteria and fungi (Table 1). The PCR reactions were conducted in a volume of 25 µl that contained 5 µl of reaction buffer (10×), 10 µl of Q-solution (5×), 1 µl of dNTP mix (10 mM), 1 µl of primer 1184-R (10 µM), 0.1 µl of primer 1184-F (10 µM), 10 ng of DNA template, and 0.25 µl of HotStarTaq DNA polymerase (5 units/µl). The Q-solution was included to aid the amplification of GC-rich templates (Qiagen, Valencia, CA). Thermal cycling parameters consisted of 5 min at 95°C, followed by 30 to 40 cycles of 45 s at 94°C, 1 min at 60°C, and 1 min at 72°C. The reaction was extended for 10 min at 72°C. Reactions were carried out using a GeneAmp PCR System 2700 (Applied Biosystems). Production of a predicted 666-bp amplicon was considered confirmation of CMN_01184 and *C. michiganensis* subsp. *nebraskensis* in a given sample.

The specificity of primer set 1184-qF2/qR2 for qPCR was evaluated using the same bacterial and fungal isolate collection that was used for the cPCR testing (Table 1). The qPCR reactions were conducted using SYBR green PCR master mix (Applied Biosystems). Reactions were conducted in a volume of 40 µl, using 0.8 µl of primer 1184-qF2 (10 µM), 0.8 µl of primer 1184-qR2 (10 µM), DNA template (50 ng), and 20 µl of Power SYBR Green PCR master mix (2×). Thermal cycling parameters were 2 min at 50°C and 10 min at 95°C, followed by 40 cycles of 15 s at 95°C, 30 s at 61°C, and 30 s at 72°C. Reactions were carried out using an ABI 7500 real-time PCR system (Applied Biosystems). Cycle threshold (C_T) values were calculated and were analyzed based on absolute quantification, using StepOnePlus software (Applied Biosystems). C_T values less than 35 were considered a positive detection response. All samples were run in duplicate on each of two separate amplification plates.

Sensitivity of cPCR and qPCR assays. Sensitivity of primer sets 1184-F/R and 1184-qF2/qR2 was evaluated using DNA template from *C. michiganensis* subsp. *nebraskensis* CIC251. Sensitivity was tested with two template sets, one represented CFU of *C. michiganensis* subsp. *nebraskensis* and the second represented pure, quantified DNA of *C. michiganensis* subsp. *nebraskensis* over a range of concentrations. For the CFU templates, *C. michiganensis* subsp. *nebraskensis* CIC395 was grown on NBY agar and the cell concentrations were adjusted to 1 × 10⁸ CFU/ml (optical density at

TABLE 2. Primers developed and used in this study for conventional polymerase chain reaction (cPCR) and quantitative PCR (qPCR) assays for *Clavibacter michiganensis* subspecies and controls

Primer	Sequence (5'-3')	Target	Type	Specificity	Source
1184-F	ATGCAATCACCTGACGATGTCGTG	CMN_01184	cPCR	<i>C. michiganensis</i> subsp. <i>nebraskensis</i>	This study
1184-R	TCAGCGACCCACATTCAGGTT				
971-F2	TCAGAGGTCCTGGGCAAAGCG	CMN_00971	cPCR	<i>C. michiganensis</i> spp.	This study
971-R1	TCCGCGACTCGCAGAACTTCG				
F27	AGAGTTTGATCMTGGCTCAG	16S rDNA	cPCR	Bacteria	Monciardini et al. 2002
R1492	TACGGYTACCTTGTTACGACTT				
1184-qF2	CATTTCCCGATATGGGCGGT	CMN_01184	qPCR	<i>C. michiganensis</i> subsp. <i>nebraskensis</i>	This study
1184-qR2	AGGGTCTTCTCGGTCAAGT				
Adh-F3	CGTCGTTTCCCATCTCTTCCTCC	<i>adh1</i>	qPCR	<i>Zea</i> spp.	Hernández et al. 2004
Adh-R4	CCACTCCGAGACCCTCAGTC				

540 nm = 0.1) in 0.85% (wt/vol) NaCl suspension, using a spectrophotometer. Ten-fold serial dilutions were made with the cell suspensions and DNA was extracted from each cell suspension as described above. Using the predicted mass of one *C. michiganensis* subsp. *nebraskensis* chromosome, the *C. michiganensis* subsp. *nebraskensis* concentration was estimated such that the dilution curve represented one to 1×10^4 *C. michiganensis* subsp. *nebraskensis* chromosomes per milliliter. For the template set based on pure DNA from *C. michiganensis* subsp. *nebraskensis*, a 10-fold serial dilution series was made by diluting a quantified DNA solution to result in concentrations ranging from 3.1 ng to 31 pg per reaction. cPCR and qPCR were conducted with both template sets, as described above. All samples were run in triplicate in three separate reactions.

***C. michiganensis* subsp. *nebraskensis* detection in soil and plant samples.** To evaluate the specificity of gene target CMN_01184 for detecting *C. michiganensis* subsp. *nebraskensis* in field soil samples, *C. michiganensis* subsp. *nebraskensis* CIC395 was grown in NBY liquid culture for 2 days and was added to soil as follows. Cell concentration was adjusted via spectrophotometry to 1×10^7 CFU/ml and was serially diluted. Sandy loam soil was collected from a maize field in Falcon Heights, MN and 0.25 g was infested with 1-ml suspensions containing 10^6 to 10^2 CFU of *C. michiganensis* subsp. *nebraskensis* per milliliter. The sampled area had no history of Goss's wilt. An additional soil sample was treated with 1 ml of sterile NBY as an untreated control. DNA was extracted from all samples, using a PowerSoil DNA isolation kit (MoBio Laboratories, Carlsbad, CA). Resultant DNA was used as template for cPCR assays with primer sets 1184-F/R and F27/R1492, as described above (Monciardini et al. 2002).

To validate the ability of the qPCR primer set 1184-qF2/qR2 to quantify and localize *C. michiganensis* subsp. *nebraskensis* populations during host infection, maize leaves exhibiting Goss's wilt symptoms were collected from four corn production fields in southern Minnesota. Whole leaves were collected from both healthy and infected corn samples and were stored in plastic bags with paper towels to inhibit moisture development during transportation. In the laboratory, all leaves were stored briefly at 4°C prior to sample extraction. Samples were collected, using a cork borer (1.8 cm²), from leaf tissue adjacent to Goss's wilt symptoms. Goss's wilt symptoms included necrosis, chlorosis, and desiccation. Tissue samples were suspended in 1.2 ml of extraction buffer as provided in the FastDNA extraction kit (MP Biomedicals, Solon, OH), and DNA was extracted according to the manufacturer's instructions. DNA was stored at -20°C. *C. michiganensis* subsp. *nebraskensis* infection was confirmed in samples exhibiting Goss's wilt symptoms via isolation on NBY.

The *C. michiganensis* subsp. *nebraskensis* concentrations in healthy and infected maize leaf samples were quantified using qPCR primer sets 1184-qF2/qR2. The maize reference gene *alcohol dehydrogenase 1* was amplified with primer sets Adh-F3/R4. In separate reactions, each sample was quantified for both *C. michiganensis* subsp. *nebraskensis* and maize DNA concentrations, using 10-fold standard curve dilution series with SYBR green, as described above, via a relative absolute quantification method. *C. michiganensis* subsp. *nebraskensis* concentrations were determined as nanograms of *C. michiganensis* subsp. *nebraskensis* DNA per gram of host DNA, and CFU was calculated based on a standard curve and the predicted chromosome mass of *C. michiganensis* subsp. *nebraskensis* NCPBP 2581 (NCBI: NC_020891.1).

RESULTS

Identification of gene targets for specific detection of *Clavibacter michiganensis* subsp. *nebraskensis* in-silico. Comparative genomics was used to identify CMN_01184 and CMN_00971 as gene targets for specific and general detection of *C. michiganensis* subsp. *nebraskensis* and all *C. michiganensis* subsp., respectively. No significant nucleic acid similarity or conserved domains were found for CMN_01184, a predicted hypothetical protein (NCBI:

YP_007685476), supporting the application of CMN_01184 as a specific target for detection of *C. michiganensis* subsp. *nebraskensis* (Table 2). Thus, CMN_01184 represents a unique and specific target for *C. michiganensis* subsp. *nebraskensis* detection. The predicted transporter CMN_00971 was identified in *C. michiganensis* subsp. *nebraskensis* and orthologs were identified in all *C. michiganensis* subspecies. No homologs were identified in other bacteria or fungi, supporting the application of CMN_00971 as a target for the detection of all *C. michiganensis* subspecies (Table 2).

Specificity and inclusivity of cPCR and qPCR assays based on CMN_01184 for detection of *C. michiganensis* subsp. *nebraskensis*. The cPCR and qPCR assays based on the primers created to target and amplify CMN_01184 were specific for the detection of *C. michiganensis* subsp. *nebraskensis*. Specificity was confirmed by evaluating a diverse panel of DNA samples from 129 bacterial and fungal isolates (Table 1). No amplification was detected with DNA from 51 isolates of 27 other bacterial and fungal maize pathogens as well as other plant- and soil-associated fungi and bacteria that were tested (Table 1). Neither cPCR nor qPCR assays based on CMN_01184 detected any of the other plant- and soil-associated bacteria and fungi, including the maize-associated pathogens *Pantoea stewartii*, *Dickeya zeae*, *Burkholderia andropogonis*, and *Exserohilum turcicum* that could confound diagnosis of Goss's wilt. Six actinomycetes were also included in our analysis. These isolates represented five families in the order Actinomycetales, including *Curtobacterium flaccumfacians*, a relative of *C. michiganensis* subsp. *nebraskensis* in the *Microbacteriaceae* family. CMN_01184 amplification was not observed with the other actinomycetes (Table 1).

To further evaluate the specificity of CMN_01184, 43 isolates representing all known subspecies of *C. michiganensis*, including subspecies *michiganensis*, *sepedonicus*, *insidiosus*, and *tessellarius* as well as the recently identified subspecies *phaseoli*, *californiensis*, and *chilensis* were evaluated (González and Trapiello 2014; Yasuhara-Bell and Alvarez 2015). None of the non-*nebraskensis* isolates amplified with CMN_01184, confirming that the cPCR and qPCR assays are specific to *C. michiganensis* subsp. *nebraskensis* and can distinguish the Goss's wilt pathogen from other *C. michiganensis* subspecies (Table 1). This extensive testing with diverse bacterial and fungal isolates demonstrated the specificity of cPCR and qPCR assays based on CMN_01184 for detection of *C. michiganensis* subsp. *nebraskensis* (Table 1). Inclusivity of the cPCR assay was confirmed by the production of the expected 666-bp amplicon corresponding to the target CMN_01184 in all 35 isolates of *C. michiganensis* subsp. *nebraskensis* tested from diverse geographical regions (Fig. 1A). All 35 isolates of *C. michiganensis* subsp. *nebraskensis* tested positive with the qPCR assays, exhibiting C_T values in the range of 14.2 to 24.5, further demonstrating high inclusivity for the assays (Table 1).

The PCR assays based on the more general primer set targeting CMN_00971 successfully amplified DNA from all isolates of all *C. michiganensis* subspecies but did not amplify DNA from any other bacterial or fungal isolates that were tested. In addition, the cPCR assays targeting CMN_00971 amplified *Clavibacter* DNA when used as the sole assay and when it was multiplexed with the *C. michiganensis* subsp. *nebraskensis*-specific assay based on CMN_01184 (Fig. 1A).

Sensitivity of cPCR and qPCR assays. The detection limits of cPCR and qPCR assays based on the CMN_01184 primer set were tested with serially diluted pure *C. michiganensis* subsp. *nebraskensis* template DNA and DNA from serially diluted viable *C. michiganensis* subsp. *nebraskensis* cells. Using pure *C. michiganensis* subsp. *nebraskensis* DNA concentrations ranging from 3 ng to 31 pg in cPCR assays, the limit of detection for the cPCR assay was ≤ 31 ng of *C. michiganensis* subsp. *nebraskensis* DNA (Fig. 1B; Table 3). Using DNA extracted from serial dilutions of *C. michiganensis* subsp. *nebraskensis* cells, the limit of detection for the cPCR assay was ≤ 100 CFU of *C. michiganensis* subsp. *nebraskensis* (Table 3). To determine sensitivity of the qPCR assay,

pure DNA was extracted from *C. michiganensis* subsp. *nebraskensis* and was diluted and quantified; DNA was extracted from cell suspensions with known numbers of *C. michiganensis* subsp. *nebraskensis* cells. CMN_01184 transcript abundance was detected with SYBR green and was analyzed using the absolute quantification method. C_T values ranged from 11.4 for 31 pg of DNA (1×10^4 *C. michiganensis* subsp. *nebraskensis* chromosomes) to 27.5 for 3.1 ng of DNA (one predicted chromosome) (Table 3). Thus, the limit of detection for the CMN_1184-based qPCR assay was ≤ 3.1 ng of pure DNA from *C. michiganensis* subsp. *nebraskensis* (Table 3). A standard curve representing the average of three replications exhibited an R^2 value of 0.99 with reaction efficiency 87.9 (Fig. 2). Efficiency was determined using Applied Biosystems 7500 Real-Time PCR software (v2.0.5). It was calculated using the slope of the standard curve regression line and the following equation: Efficiency = $(10^{-1/\text{slope}} - 1) \times 100$. Sensitivity of the assays in terms of *C. michiganensis* subsp. *nebraskensis* cell number was also determined using DNA extracted from serial dilutions of *C. michiganensis* subsp. *nebraskensis* cultures. The standard curve represented 1×10^4 CFU/ml, and using the absolute quantification method produced C_T values ranged from 19.9 to 34.1, demonstrating a lower limit of detection of 10 CFU (Table 3).

Detection and quantification of *C. michiganensis* subsp. *nebraskensis* in infected maize leaves and soil samples. The efficacy and robustness of the cPCR and qPCR assays based on CMN_01184 for detecting and quantifying *C. michiganensis* subsp. *nebraskensis* in plant samples were evaluated with maize leaves with symptoms of Goss's wilt and with asymptomatic maize leaf tissue collected at four field locations in Minnesota. Multiplexed cPCR assays based on the *C. michiganensis* subsp.

nebraskensis-specific and *C. michiganensis*-general primer sets 1184-F/R and 971-F2/R1, respectively, resulted in the production of amplicons only with DNA extracts from symptomatic tissues with both primer sets. No amplicons were produced from the asymptomatic leaf tissue (Fig. 1C). *C. michiganensis* subsp. *nebraskensis* was also detected and quantified only in infected maize leaves with the qPCR assay. *C. michiganensis* subsp. *nebraskensis* concentrations in maize samples ranged from 5.3×10^8 to 9.3×10^9 CFU ml⁻¹ per sample (Table 4). These results indicate that the qPCR assay based on CMN_01184 can effectively quantify populations of *C. michiganensis* subsp. *nebraskensis* in infected leaves and measure the development of infection. Further, the microbiome of healthy maize leaves did not create false positives or inhibit detection of *C. michiganensis* subsp. *nebraskensis* with the PCR assays.

The ability of the PCR assays based on CMN_01184 to detect *C. michiganensis* subsp. *nebraskensis* in soil was evaluated with DNA extracted from field soil containing a range of added concentrations of viable *C. michiganensis* subsp. *nebraskensis*. Using cPCR, *C. michiganensis* subsp. *nebraskensis* was only amplified in the soil samples infested with *C. michiganensis* subsp. *nebraskensis* (Fig. 3). The cPCR assay detected *C. michiganensis* subsp. *nebraskensis* at concentrations as low as 400 CFU per cubic centimeter of soil (Fig. 3). To evaluate whether PCR inhibitors in the soil could have influenced amplification, DNA was extracted from soil infested or not with *C. michiganensis* subsp. *nebraskensis* and the primer set F27/R1492 was used to amplify DNA from all bacteria. A 1.5-kb amplicon indicative of 16S bacterial rDNA amplification was produced with all soil samples (Fig. 3). These results demonstrate that PCR assays based on CMN_01184 are robust and specific for detection of *C. michiganensis* subsp. *nebraskensis* in complex soil environments.

DISCUSSION

Here, we report the development and validation of a novel DNA target and PCR assays for specific detection and quantification of

Fig. 1. Specificity and sensitivity of the gene CMN_01184 as a target for in vitro and in planta detection of *Clavibacter michiganensis* subsp. *nebraskensis* as determined via conventional polymerase chain reaction (PCR). **A**, Amplification of CMN_01184 (666 bp, top band) distinguishes *C. michiganensis* subsp. *nebraskensis* from other *C. michiganensis* subspecies, while CMN_00971 (390 bp, lower band) is conserved in all *C. michiganensis* subspecies tested including *michiganensis* (*Cmm*), *sepedonicus* (*Cms*), *insidiosus* (*Cmi*), and *tessellarius* (*Cmt*). **B**, Sensitivity of conventional PCR for detection of pure DNA from *C. michiganensis* subsp. *nebraskensis*. The assay based on the CMN_01184 primers detected ≤ 10 pg of *C. michiganensis* subsp. *nebraskensis* DNA. **C**, Detection of *C. michiganensis* subsp. *nebraskensis* from maize tissues exhibiting symptoms of Goss's wilt collected from four Minnesota field locations (right four lanes); no *C. michiganensis* subsp. *nebraskensis* was detected in DNA isolated from asymptomatic maize tissue (second through fifth lanes).

TABLE 3. Sensitivity of conventional polymerase chain reaction (cPCR) and quantitative qPCR detection assays based on gene CMN_01184, using pure DNA and whole cells of *Clavibacter michiganensis* subsp. *nebraskensis*

Template ^a	PCR detection	
	cPCR ^b	qPCR (C_T) ^c
31 pg <i>C. michiganensis</i> subsp. <i>nebraskensis</i> DNA	+	11.7 $\pm$ 0.6
3.1 pg <i>C. michiganensis</i> subsp. <i>nebraskensis</i> DNA	+	15.8 $\pm$ 0.7
0.31 pg <i>C. michiganensis</i> subsp. <i>nebraskensis</i> DNA	+	19.4 $\pm$ 0.9
3.1×10^{-2} pg <i>C. michiganensis</i> subsp. <i>nebraskensis</i> DNA	+	23.9 $\pm$ 1.1
3.1×10^{-3} pg <i>C. michiganensis</i> subsp. <i>nebraskensis</i> DNA	–	27.5 $\pm$ 0.9
10,000 CFU <i>C. michiganensis</i> subsp. <i>nebraskensis</i>	+	19.9 $\pm$ 0.1
1,000 CFU <i>C. michiganensis</i> subsp. <i>nebraskensis</i>	+	23.4 $\pm$ 0.0
100 CFU <i>C. michiganensis</i> subsp. <i>nebraskensis</i>	+	26.9 $\pm$ 0.0
10 CFU <i>C. michiganensis</i> subsp. <i>nebraskensis</i>	–	30.5 $\pm$ 0.4
1 CFU <i>C. michiganensis</i> subsp. <i>nebraskensis</i>	–	34.1 $\pm$ 0.7

^a Templates represent either quantified pure *C. michiganensis* subsp. *nebraskensis* DNA or DNA isolated from quantified serial dilutions of *C. michiganensis* subsp. *nebraskensis* culture; 3.1×10^{-3} pg represents the predicted mass of one *C. michiganensis* subsp. *nebraskensis* chromosome.

^b + = expected 0.67-kb PCR product band; – = no PCR product band detected.

^c Cycle threshold (C_T) values from qPCR reactions are mean values and standard deviations from samples run in triplicate in three separate reactions. Samples with C_T values greater than 35 were considered to have nondetectable *C. michiganensis* subsp. *nebraskensis* DNA.

C. michiganensis subsp. *nebraskensis*. The cPCR and SYBR green-based qPCR assays should be readily accessible and easily adopted in many laboratories for diagnosis of and research on Goss's wilt. Both PCR assays are sensitive tools for detection of *C. michiganensis* subsp. *nebraskensis* in plant and soil samples and can distinguish *C. michiganensis* subsp. *nebraskensis* from other *C. michiganensis* subspecies in addition to a diverse array of other maize and plant-associated bacteria and fungi.

Using comparative genomic approaches, CMN_01184 was identified in the genome of *C. michiganensis* subsp. *nebraskensis* NCPPB 2581 as a putative singleton and primers were created for cPCR and qPCR amplification. CMN_01184 was confirmed as a specific target for the detection of *C. michiganensis* subsp. *nebraskensis* by evaluating a collection of 129 bacteria, including 78 isolates of *C. michiganensis* and a diverse array of fungi isolates. These isolates included numerous bacterial and fungal pathogens of maize and the pathogenic *C. michiganensis* subspecies *michiganensis*, *sepedonicus*, *insidiosus*, and *tessellarius*. The maize fungal pathogens *Exserohilum cloacae* and *Fusarium graminearum* were tested along with the widespread maize bacterial pathogens *Dickeya zeae*, *Pantoea stewartii*, and *Burkholderia andropogonis*. PCR assays based on CMN_01184 were specific to *C. michiganensis* subsp. *nebraskensis* in all cases, and this gene target was not detected in maize pathogens or other fungi or bacteria or in plant and soil samples tested in this study.

C. michiganensis represents a species of pathogenic bacteria with a diverse and large host range. Since 2014, three new *C. michiganensis* subspecies have been identified, i.e., *phaseoli*, *chilensis*, and *californiensis*. As additional *C. michiganensis* subspecies are confirmed, it is important that detection assays are specific and capable of

differentiating between all known subspecies. Currently, the most common strategies for detection of *C. michiganensis* subsp. *nebraskensis* and diagnosis of Goss's wilt employ agar-based media and commercially available serological assays that cannot distinguish *C. michiganensis* subsp. *nebraskensis* from subspecies *michiganensis* or *tessellarius* or several other common bacteria found in agricultural fields (Korus 2011). Here, we report, for the first time, novel cPCR and SYBR green-based qPCR assays targeting the gene CMN_01184, which can distinguish *C. michiganensis* subsp. *nebraskensis* from all known *C. michiganensis* subspecies as well as a diverse array of other pathogens.

Effective assays for detection and quantification of pathogens must be both specific and sensitive. To demonstrate the sensitivity of our qualitative and quantitative detection assays, *C. michiganensis* subsp. *nebraskensis* DNA samples and cell suspensions were tested. Our cPCR assay could detect 3.1×10^{-2} pg of DNA template and 100 CFU of *C. michiganensis* subsp. *nebraskensis*. The qPCR assay had a lower limit of detection of ≤ 3.1 ng of pure *C. michiganensis* subsp. *nebraskensis* DNA template and 10 CFU of *C. michiganensis* subsp. *nebraskensis*. This level of sensitivity is excellent for diagnosis and for many types of infection, ecological, and epidemiological studies.

C. michiganensis subsp. *nebraskensis* is a systemic vascular pathogen believed to overwinter in infected maize residues. To directly evaluate the utility of *C. michiganensis* subsp. *nebraskensis* PCR detection assays based on CMN_01184 for studies of pathogen ecology and epidemiology, *C. michiganensis* subsp. *nebraskensis* was detected directly from both multiple-plant and soil samples infected with *C. michiganensis* subsp. *nebraskensis*. In all cases, *C. michiganensis* subsp. *nebraskensis* was detected successfully only from maize tissues exhibiting Goss's wilt symptom and was

Fig. 2. Standard curve representing quantitative amplification of target gene CMN_01184 in *Clavibacter michiganensis* subsp. *nebraskensis*. The relationship between picograms of pure DNA from *C. michiganensis* subsp. *nebraskensis* and cycle threshold (C_T) values from the quantitative polymerase chain reactions is shown. Trend-line data points represent the mean of three replications. Error bars represent standard deviation.

TABLE 4. Quantification of *Clavibacter michiganensis* subsp. *nebraskensis* from infected maize leaf samples^a

Location	<i>C. michiganensis</i> subsp. <i>nebraskensis</i>		Maize	<i>C. michiganensis</i> subsp. <i>nebraskensis</i> /maize	
	DNA (ng) ^b	CFU ml ^{-1c}	DNA (ng) ^b	DNA (ng/ng) ^b	Titer (CFU/ng) ^b
Martin	21.58	3.0×10^9	260.0	0.1	2.6×10^4
Watsonwan	85.16	9.3×10^9	71.0	1.2	3.8×10^5
Hastings	41.51	3.9×10^9	192.1	0.2	6.9×10^4
Rosemount	12.68	5.3×10^8	28.3	0.4	1.4×10^5

^a Maize leaf samples were collected from four Minnesota field locations, using a quantitative polymerase chain reaction assay based on amplification of CMN_01184 and quantification of the maize reference gene *adh1*. The *adh1* primers were developed by Hernández et al. (2004).
^b Concentration of *C. michiganensis* subsp. *nebraskensis* and maize (*Zea mays* subsp. *mays*) DNA was determined via absolute quantification, using the total genomic DNA standard curve method in a 40-μl reaction.
^c CFU represents a predicted value based on the standard curve of cycle threshold value versus quantity of *C. michiganensis* subsp. *nebraskensis* DNA and the mass of the *C. michiganensis* subsp. *nebraskensis* chromosome (3.13 fg).

not detected in any asymptomatic maize leaves. Furthermore, to confirm the specificity and sensitivity of CMN_01184, soil samples were infected with *C. michiganensis* subsp. *nebraskensis* and were tested using our cPCR assay. *C. michiganensis* subsp. *nebraskensis* was detected only in soil samples infected with *C. michiganensis* subsp. *nebraskensis* and our reaction controls amplified general 16S bacterial DNA in all soil samples, regardless of *C. michiganensis* subsp. *nebraskensis*–status, confirming that DNA extractions were successful and PCR inhibitors were not significantly influencing the PCR reactions. Collectively, these results demonstrate, for the first time, that PCR assays based on CMN_01184 are specific and sensitive tools to detect and quantify *C. michiganensis* subsp. *nebraskensis* in plant and soil samples. Further, other microbes in maize leaves and field soil do not create false-positive results or interfere with detection of *C. michiganensis* subsp. *nebraskensis*.

For research on the epidemiology and host-pathogen interactions of *C. michiganensis* subsp. *nebraskensis*, a precise tool for determining *C. michiganensis* subsp. *nebraskensis* population levels in planta during host infection will be valuable. Current strategies for *C. michiganensis* subsp. *nebraskensis* quantification are nonspecific and require time-consuming plating techniques susceptible to contamination from other host-associated microbial organisms (Smidt and Vidaver 1986). Using infected maize samples collected from multiple maize production fields, *C. michiganensis* subsp. *nebraskensis* populations could be quantified and enumerated relative to host DNA concentrations with the new qPCR assay. Importantly, these results validate, for the first time, a molecular approach for the elucidation of *C. michiganensis* subsp. *nebraskensis* cell concentration, infection, and localization during host infection. The use of *adh1* as a maize reference gene accounted for potential PCR inhibitors and differential DNA loss during the isolation process to ensure accurate results.

In summary, we report for the first time a pair of comprehensively tested PCR assays for detection and quantification of *C. michiganensis* subsp. *nebraskensis* based on the unique gene target CMN_01184. No previously reported techniques were available for both specific detection and quantification of *C. michiganensis* subsp. *nebraskensis* that utilized relatively inexpensive and easily adopted cPCR and SYBR green qPCR assays. Thus, we have addressed the need to develop

and validate a cPCR assay that is a simple, cost-effective tool for rapid diagnosis and other applications, as well as a qPCR assay that is highly sensitive, quantitative, and cost effective and has high throughput potential for diagnosis and research on the epidemiology, ecology, and host-pathogen interactions of *C. michiganensis* subsp. *nebraskensis*.

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Fig. 3. Conventional polymerase chain reaction (PCR) detection of *Clavibacter michiganensis* subsp. *nebraskensis* using CMN_01184-specific primers (Cmn-specific) in field soil samples infested with 0, 10⁴, or 10⁶ CFU of *C. michiganensis* subsp. *nebraskensis* per milliliter. Bacterial 16S rDNA (1.5 kb) was also amplified using primer pair F27 and R1492 to check for successful DNA isolations and for the presence of PCR inhibitors. *C. michiganensis* subsp. *nebraskensis* was detected only in soil samples containing *C. michiganensis* subsp. *nebraskensis* DNA, and 16S bacterial rDNA was detected in all soil samples, as expected.

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